(54) Title: ERBB2 PROMOTER BINDING PROTEIN IN NEOPLASTIC DISEASE

(57) Abstract

The present invention provides a purified and isolated DNA-binding protein, HPBF, which specifically binds to the promoter region of the Her-2/*neu* (ERBB2/c-*erb*B-2) gene sequence, the presence of which provides an early indication of transition to a cancerous state has been found. The present invention also provides bioassays for screening substances for the ability to inhibit HPBF activity, the ability to inhibit the mitogenic activity of HPBF and the ability to inhibit HPBF production. The present invention further provides methods of inhibiting the biological activity mediated by HPBF comprising preventing the HPBF from binding to the promoter region of the ERBB2 gene sequence.

1

ERBB2 PROMOTER BINDING PROTEIN
IN NEOPLASTIC DISEASE

BACKGROUND OF THE INVENTION

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FIELD OF THE INVENTION

The present invention relates generally to the field of medical diagnosis and specifically for monitoring the presence of neoplastic diseases at an early stage to allow early therapeutic intervention.

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BACKGROUND ART

Currently, early detection of breast cancer in humans, particularly in women, depends on self-examination and mammography. However, routine mammography is not recommended for women under 50. Therefore, breast cancers in
15   younger women tend not to be found until more advanced with a correspondingly poorer prognosis. Screening methods are needed to identify early stages of the transition of normal epithelial cells towards carcinoma *in situ* before the subsequent development of invasive and metastatic cancer.

20   Breast cancer appears to be genetically and/or morphologically, a heterogeneous disease and multiple mechanisms are responsible for the ultimate development of breast carcinoma from normal epithelial cells. The Her-2/*neu* (ERBB2/c-*erb*B-2) gene sequence (SEQ ID NO:9), hereinafter referred to as ERBB2, appears to be one of the primary genes responsible for the transition of normal epithelial
25   cells towards carcinoma *in situ* and the subsequent development of invasive and metastatic cancer. However, by the time the gene product of ERBB2 is measurable, prognosis is not good. A means of identifying the initiation step for ERBB2 gene activity and interfering with that step are necessary for greater success in early identification and treatment of breast cancer.

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Significant progress has been made at the molecular level to dissect the role of the ERBB2 gene and its association with breast cancer. However, mechanisms that control or initiate the activity of the ERBB2 gene have not been available to give early prediction or treatment of breast cancer. The results of some of these molecular

5   studies are described herein.

Histologically, breast cancer comprises about 70-85% classified as ductal carcinoma; the next largest subgroup is referred to as lobular carcinoma. These two major classes of breast cancer comprise more than 80-95% of breast cancer in humans.

·10   It has been estimated that 5-15% of breast cancer in women under 50 years of age is associated with a genetic propensity for the disease.[1-13]   Several recent studies have elucidated some of the inherited mechanisms which are at work in breast cancer.[14-17] A recent review has described various molecular determinates of growth, angiogenesis and metastases which may play a role in breast cancer.[18] In addition, the ERBB2 gene has

15   recently been documented to be prognostically important in breast cancer.[43,45,56,69]

The ERBB2 gene is the human counterpart of the rat *neu* oncogene (SEQ ID NO:12), originally identified in ethyl nitroso-urea induced rat neuroglioblastomas by Weinberg and co-workers.[19,20] The ERBB2 oncogene codes for

20   a protein of 185,000 dalton molecular weight (p185 product), and the product is similar in overall organization and primary amino acid sequence to the epidermal growth factor receptor (EGFR).[21-23] A possible ligand for ERBB2 has recently been described.[24-26] The ERBB2 gene is not overexpressed in benign breast tissue,[27] but significantly overexpressed in 60% of carcinoma *in situ* (preneoplastic lesion of breast carcinoma)

25   and in about 30% of invasive cancer.[28-30]

The p185 product of the ERBB2 gene is a growth factor receptor with intrinsic protein tyrosine kinase activity [31,32] which, when deregulated, or disregulated, results in unrestrained growth and cell transformation.[32-34] The transforming potential

30   of the ERBB2 gene is also related to the levels of protein expression. This proto-oncogene is also frequently amplified in many human tumors and in cell lines

3

derived from tumors.[33,35-38] ERBB2 gene overexpression in the absence of gene amplification has also been described.[33,36-38] The ERBB2 gene product is a potent oncoprotein when overexpressed in NIH-3T3 cells.[34] In a transgenic mouse model experiment, transgenic mice were created[39,40] expressing the activated form of the rat

5   *neu* proto-oncogene, under the control of steroid inducible promoter, and uniformly developed mammary adenocarcinoma. In addition, ERBB2 gene amplification in human breast tumor is often associated with poor patient prognosis.[33,38] The overexpression of ERBB2 has also been associated with poor prognosis in non-small cell lung cancer.[41,85]

·10   A convincing body of clinical and experimental evidence thus supports the role of ERBB2 protein in the progression of human cancers characterized by the overexpression of this oncogene product. Important aspects of this evidence include the poor prognosis of breast, ovarian and non-small cell carcinoma patients whose tumors overexpress ERBB2 protein, as well as observations which indicate that modulation of

15   ERBB2 protein activity by a monoclonal antibody can reverse many of the properties associated with tumor progression mediated by growth factor receptor.[42]

             A recent study[43] of 209 consecutive female patients with invasive operable breast cancer from a defined urban population observed for a median of 30

20   years demonstrated that fifty-five patients (26%) had cancer and a positive ERBB2 oncoprotein stain reaction. They had significantly reduced 10 and 25 years survival rates as compared with those patients who had a negative stain reaction in their cancer (31% versus 48% and 31% versus 39% respectively with a P value = 0.004). ERBB2 gene expression was also found to be associated with reduced survival among patients

25   who had axillary nodal metastases (P value = 0.003) but not among those patients who did not have metastases. ERBB2 expression was related to the ductal histologic type, poor histologic grade and high mitotic count, but not to tumor size, axillary nodal status, DNA ploidy or S-phase fraction. In a multivariate analysis among patients with nodal metastases, ERBB2 expression was found to be an independent prognostic factor (P

30   value = 0.004) that predicted poor survival. Based on these data, it was concluded that ERBB2 oncoprotein expression has long-term prognostic significance for predicting

4

poor survival in breast cancer and it has an independent prognostic value among patients who presented with axillary nodal metastases. The mean survival time for the women with ERBB2 expressing group is only 29 months compared to the mean survival time of 110 months of the women with nonexpressing cancer. The difference between the

5   survival curve is the greatest at approximately five years from the diagnosis (37% versus 64%) and diminished toward the end of the follow-up, which indicates that ERBB2 expressing cancers usually progress rapidly and are fatal. The result that ERBB2 expression predicts poor survival is contradictory to the opinion that it could only be a marker for drug resistance,[44] not a marker for poor prognosis.

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Overexpression of the ERBB2 oncogene has previously been correlated with poor prognosis in patients with infiltrating breast carcinoma.[33] The authors reported a 35% difference in survival at four years for node positive patients with ERBB2 positive tumors.[33] This finding was emphasized in later studies with large

15   numbers of patients.[45] It appears that the inconsistencies in the relationship between ERBB2 overexpression and mammary carcinoma are related to its correlation with tumor type. In studies of infiltrating carcinoma, the proportion of tumors showing overexpression has ranged from 10-30%;[28-30,33,46-47] in carcinoma *in situ*, the incidence of overexpression is much higher, in the order of 60%.[28-30]

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Several studies[45,48-50] have clearly shown that there is no loss of ERBB2 expression when invasive tumors progress from a pure *in situ* carcinoma. Therefore, there must be some other reason why fewer infiltrating tumors overexpress ERBB2. The nuclear sizes of the *in situ* and infiltrating components were also very similar and as

25   has been found previously for *in situ* disease, almost all of the ERBB2 positive cases contained some large nuclei. A study[51] has suggested that there are at least three groups of infiltrating tumors:

Group 1 - those composed of cells with small nuclei which have arisen

30   from small cell cribriform/micropapillary ductal carcinoma *in situ*. These have a low rate of proliferation and of ERBB2 overexpression.

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Group 2 - tumors composed of large cells which have arisen from large cell comedo ductal carcinoma *in situ*. These have a high rate of proliferation and ERBB2 overexpression.

5   Group 3 - tumors composed of cells with variable nuclear sizes, but including some large nuclei, over half of which have a high rate of proliferation, but none of which overexpress ERBB2.

The hypothesis is that the latter group of tumors only have a transient *in situ* period and quickly become invasive. Because of this rapid progression to invasion, these tumors were not found in these studies of pure ductal carcinoma *in situ*. They made only a minor contribution to that study of tumors with a prominent ductal carcinoma *in situ* component accompanied by a variable infiltrating component but have become very obvious in this particular study. This could explain the dilution of overall ERBB2 positivity seen in studies of infiltrating tumors when compared to pure *in situ* tumors. If this is so, it could be accepted that the presence of ERBB2 overexpression is a marker of poor prognosis, since the ERBB2 positive *in situ* tumors are always composed of large cells, usually of comedo pattern and there are data to suggest that such tumors have a greater invasive potential than other patterns of *in situ* carcinoma.[52-55] In cases of infiltrating carcinoma, the ERBB2 positive tumors again contain large cells and are rapidly proliferating, both factors being associated with a poor prognosis. Whereas tumors with small nuclei and tumors with low proliferative activity are nearly always ERBB2 negative, there are also significant numbers of ERBB2 negative tumors which contain at least some large cells, and many of these tumors have a high rate of proliferation. As already suggested, it is possible that this group of tumors has only a transient *in situ* stage.

Finally, another recent study[56] demonstrated that tumors from 16% of the node negative patients and 19% of the node positive patients were ERBB2 positive. In both groups, ERBB2 positively correlated with negative progesterone receptor, negative estrogen receptors and high tumor grade. The expression of ERBB2 was

6

prognostically significant for node positive, but not for node negative patients. Tumors
with overexpression of ERBB2 oncogene were less responsive to cyclophosphamide
methotrexate and fluorouracil containing adjuvant therapy regimens than those with a
normal amount of gene product, suggesting worse tumor behavior. For node positive

5   patients, the effect of prolonged duration therapy on disease free survival was greater
for patients without ERBB2 overexpression than those with ERBB2 overexpression.
Similarly, for node negative patients, the effect of perioperative treatment on disease
free survival was greater for those without ERBB2 overexpression than for those with
ERBB2 overexpression.

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United States Patents 4,935,341 to Bargmann *et al.*, issued June 19,
1990, 4,968,603 to Slamon *et al.* issued November 6, 1990 and 5,183,884 to Kraus *et
al.*, issued February 2, 1993, provide methods relating to the identification of ERBB2
gene expression, overexpression and prognostic indicators of breast cancer based on the

15   ERBB2 gene product. The Slamon *et al.* '603 patent discloses amplification of the
ERBB2 oncogene and its relationship to the status of breast and ovarian
adenocarcinomas. In particular, the degree of gene amplification provides prognostic
utility for breast cancer. The Bargmann *et al.* '341 patent discloses mutations in the
ERBB2 gene which result in an oncogenic state and provide an oligonucleotide probe

20   capable of hybridizing to the mutated region. The Kraus *et al.* '884 patent discloses a
DNA fragment distinct from EGFR and the ERBB2 gene, designated as ERBB-3.
Marked elevation of ERBB-3 mRNA levels were demonstrated in certain human
mammary tumor cell lines.

25   The above research and patents do not provide information that allows
screening to identify earlier stages of the transition of normal epithelial cells towards
carcinoma *in situ* before the subsequent development of invasive and metastatic cancer.
These results indicate that the ERBB2 gene is extremely important in a significant
percentage of breast cancers and the regulation of expression is perhaps a key

30   determining factor in breast cancer development and progression. If the regulation can
be controlled, transition to a cancerous state can be stopped.

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Recent studies of cloning and characterization of an ERBB2 promoter have compared mouse *neu* promoter (SEQ ID NO:15) with human ERBB2 promoter.[57] (SEQ ID NO:10; SEQ ID NO:11) The presence of CAAT box and lack of a TATAA motif is one way in which the mouse *neu* promoter differs from the human ERBB2

5   promoter[58] but is similar to the rat *neu* promoter.[59] (SEQ ID NO:13; SEQ ID NO:14) The GGA repeats observed between -204 and -184 (with respect to the translational start "ATG" codon) of the mouse *neu* promoter are also seen in rat[59] *neu* and human ERBB2 promoters.[58] A sequence consensus for SP1 is located at -211 of the mouse *neu* promoter. SP1 consensus sequences are also seen in rat *neu* promoter and the

10   human ERBB2 promoter in an analogous region. The sequence GCCGCCGC at -140 in the mouse *neu* promoter is similar to the binding site for G-CSF[60] and is also observed in the rat *neu* promoter but not in the human ERBB2 promoter. A sequence similar to the OTF 1 motif, [61,62] but differing by one nucleotide (ATGCAAAC instead of ATGCAAAT), is located at position -462. A similar sequence is also seen in the rat *neu*

15   promoter and human ERBB2 promoters at equivalent positions. Sequences with homology to the AP2 consensus sequence (T/CC/GC/GCCA/CNG/CC/GG/C)[63] are located at -328 and -106 of the mouse *neu* promoter gene; similar sequences are also found in the corresponding regions of the rat *neu* promoter and human ERBB2 promoter.

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A novel transcription factor termed "RNF"[64] was found to bind to the promoter of the rat *neu* gene. The binding sequence for this factor is also present in both the mouse (-439) *neu* promoter and human ERBB2 promoter. The GGTGGGGGGG sequence, termed "GTG" enhancer, which is involved in

25   autorepression of the rat *neu* transcription [59] is located at position -249 to -240 in the mouse *neu* promoter. However, the corresponding region of the human ERBB2 promoter is different. Conservation of transcription factor sequences among these three species may imply a conserved function. It is not known at the present time whether those sequences that are different between rodent and human genes such as CAAT and

30   TATAA box, GTG enhancer and other motifs might represent species specific functions.

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This information, together with the fact that multiple transcriptional initiation sites are mapped in both the rat *neu* and human ERBB2 genes, makes it likely that the TATAA sequence in the human ERBB2 promoter does not function as a transcriptional TATAA box. The previous studies on rat *neu* and human ERBB2

5   promoters focused mainly on a region within 1 Kb upstream from the transcriptional initiation sites. The current studies on the mouse *neu* promoter[57] have lead to identification of a silencer region approximately three Kb upstream from the transcriptional initiation site, similar sequences have not yet been reported in human ERBB2 promoter. An estrogen responsive region has been found within the rat *neu*

10   promoter region.[70]

It has been reported that the expression of the ERBB2 gene is tissue specific and developmentally regulated.[65] Transcriptional regulation, therefore, may be one of the mechanisms (factor) leading to overexpression of ERBB2 gene in human

15   cancer cells. Therefore, regardless of the relative distances from the transcriptional initiation site, identification of silencer and enhancer sequences controlling ERBB2 transcription provides important information that may allow clinical information to be obtained for studying transcriptional mechanisms resulting in cancer and understanding the biological role of ERBB2 gene regulation in breast cancer development,

20   heterogeneity, progression and recurrence.

Primary gene induction or repression in eukaryotes does not require *de novo* protein synthesis, suggesting the involvement of post-translational modifications as well. In a recent review,[67] it was summarized that many different types of stimuli that

25   affect gene expression also led to the activation of protein kinases; it is likely that transcription factor function will be directly regulated by phosphorylation. Even though other types of post-translational modifications will undoubtedly be important in regulating transcription factor function, phosphorylation seems to be one of the most important functions which has been studied recently.[67-68]

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In summary, first, a transcription factor can be sequestered in the

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cytoplasm and rendered inactive through lack of access to the target sequences. Phosphorylation of the factor itself, or a cytoplasmic anchor protein allows translocation of the transcription factor into the nucleus, where it acts, generally by binding to the DNA at a specific site by protein-DNA interaction.[73] Second, the DNA-binding activity

5   of nuclear transcription factor can be modulated by phosphorylation either positively or negatively.[67-68] Third, phosphorylation can affect the interaction of transcription factor transactivation domains with the transcriptional machinery.[67-68] These possibilities are by no means mutually exclusive and in principle phosphorylation at multiple sites by different protein kinases can result in regulation at several distinct levels. Nuclear

10   translocation of various transcription factors modulated by phosphorylation has been demonstrated recently.[72]

It has been shown that in unstimulated cells, with the notable exception of B cells, NFκB (nuclear factor κB) is retained in the cytoplasm in an inactive complex with the intermediary protein (IκB), which cannot bind DNA.[73,74] In response to

15   various stimuli, including the phorbol-ester TPA, the IκB-NFκB complex dissociates and NFκB DNA-binding activity is detected in the nucleus.[73] DNA binding activity can be revealed in unstimulated cytoplasmic extracts by a number of means including treatment with sodium deoxycholate, which dissociates the IκB-NFκB complex.[74]

20   Therefore, there is much evidence to suggest that a transcription factor can be found in the cytoplasmic extracts, as well as in the nuclear extract.[67] A phosphorylation-dephosphorylation mechanism for the translocation of transcription factor in numerous systems by protein kinase A and protein kinase C has been demonstrated as indicated earlier.[67-68] Almost every eukaryotic transcription factor that

25   has been analyzed in detail has proved to be phosphorylated. In most cases, however, the functional consequences of such phosphorylations, if any, are largely unknown.

There are only a few possible mechanisms proposed for the regulation of ERBB2 gene expression which are summarized as follows:

30   (i) A recent report has suggested that the E3 region of adenovirus induces down regulation of epidermal growth factor receptor. A similar repression of ERBB2

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expression has also been documented, however, the repressed expression of ERBB2 is not through the E3 region of the adenovirus. The repression of ERBB2 expression is accomplished by E1A gene product, and it specifically repressed ERBB2 gene expression at the RNA level[75] and full basal promoter activity of ERBB2 gene has been

5 shown to be retained by two fragments of the ERBB2 5' region (-759 to-724 and -396 to -24 base pair).

(ii) Functional inactivation of both alleles of the retinoblastoma susceptibility gene (RB) plays an important role in the etiology of both sporadic and familial retinoblastomas and several other types of human cancers, including breast cancer.[76,77]

10 The RB gene may have cell cycle control function.[78,79] RB protein function may vary during the cell cycle because it shows cell cycle dependent changes in phosphorylation and RB protein can be phosphorylated by the cell cycle kinase p34 cdc2.[80] RB protein can also complex with the transcription factor E2F and inhibit E2F binding to the promoters of several cellular proliferation related genes.[81] Recent studies revealed that

15 RB protein can negatively regulate the immediate early genes of *c-fos* and *c-myc* expression at the transcriptional level in NIH-3T3 cells.[82,83] RB also stimulates the growth inhibitory factor TGF-β1 expression in certain cell types and subsequently suppresses cell growth.[84] Taken together, all of these results suggest that RB may limit the progression of cells through the cell cycle by sequestering a variety of nuclear

20 proteins involved in growth regulatory gene transcription. As indicated earlier the amplification and overexpression of ERBB2 is involved in human breast and lung cancers.[38,85] Interestingly, inactivation of the RB gene has also been implicated in the oncogenesis of human breast and lung cancers[77,86] and may suggest the possible molecular link between RB and the ERBB2 gene in the development and progression of

25 breast cancer. A recent study has shown that the RB protein can bind specifically with a GTG-GGGGGGG sequence in the ERBB2 promoter and suppress the promoter function. This study has concluded that the RB protein suppresses ERBB2 induced transformation by suppressing the ERBB2 promoter activity.[87]

(iii) An interesting feature of the human ERBB2 gene promoter is the presence

30 of two different types of regulatory elements: a CAAT box and SP1 binding sites. Transcription from the three most downstream RNA start sites appear to be controlled

by the CAAT box and the TATA box, because these are respectively about 30 bp and 80 bp upstream of the early start sites and these distances are consistent with those in many other eukaryotic promoters.[88] On the other hand, transcription from the fourth RNA start sites located further upstream seems to be controlled at least partly by SP1.

5   In contrast with the ERBB2 gene promoter, the promoter region of the human epidermal growth factor receptor (EGFR) gene does not contain either a TATA box or a CAAT box but has 5 SP1 binding sites. Therefore, the expression of the ERBB2 gene may be regulated by the transcription factor SP1, a CAAT box binding protein and a TATA box binding protein,[89-91] whereas the expression of the EGFR gene seems to be

10   regulated by SP1 but not by the latter two proteins.

          Since the ERBB2 gene appears to be important in breast cancer, treatment modalities have been reported in the literature employing strategies which target this gene. A recent report[71] used a monoclonal antibody coupled to a toxin to

15   target the extracellular domains of the ERBB2 receptor protein which are overexpressed on human breast and ovarian tumor cells *in vitro*. However, this is again late in the stage of the transition of normal epithelial cells to cancer. As described earlier, ERBB2 expressing cancers usually progress rapidly and are fatal. Treatment and diagnosis needs to be at an earlier stage, while the cells are still only showing hyperplasia.

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                              SUMMARY OF THE INVENTION

          The present invention provides a purified and isolated DNA-binding protein which specifically binds to the promoter region of the c-*erb*B-2 gene sequence

25   (Her-2/*neu* promoter binding factor: HPBF).

          The present invention also provides antibodies which specifically bind HPBF. The present invention further provides a bioassay for determining the amount of HPBF in a biological sample comprising contacting the biological sample with a nucleic

30   acid or antibody to which the HPBF binds under conditions such that an HPBF/nucleic acid complex or an HPBF/antibody complex can be formed and determining the amount

12

of the complex, the amount of the complex indicating the amount of HPBF in the sample.

The present invention also provides a method of detecting the presence of a cancer in a subject and determining the prognosis of a subject having cancer comprising determining the presence of a detectable amount of HPBF in a biopsy from the subject, the presence of a detectable amount of HPBF, relative to the absence of HPBF in a normal control indicating the presence of cancer and a decreased chance of long-term survival.

The present invention further provides a DNA isolate encoding HPBF.

In addition, the present invention provides a bioassay for screening substances for ability to inhibit the activity of HPBF comprising administering the substance to a cell construct comprising the promoter region of ERBB2 linked to a reporter gene and an activated gene encoding HPBF and determining the amount of the reporter gene product and selecting those substances which inhibit the expression of the reporter gene product.

The present invention also provides a bioassay for screening substances for the ability to inhibit the mitogenic activity of HPBF in NIH3T3 cells comprising administering the substance to the cells, administering HPBF to the cells, determining the mitogenic activity of HPBF in the substance-treated cells and selecting those substances which inhibit the mitogenic activity of HPBF in the cells.

The present invention further provides a bioassay for screening substances for the ability to the inhibit the production of HPBF comprising administering the substance to a cell having an activated gene encoding HPBF and determining the amount of HPBF produced and selecting those substances which inhibit the production of HPBF.

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Finally, the present invention provides a method of inhibiting a biological
activity mediated by HPBF comprising preventing the HPBF from binding to the
promoter region of the ERBB2 gene sequence wherein the binding to the promoter
region is prevented by an antisense nucleotide sequence or wherein the binding to the
5   promoter region is prevented by a nongenomic nucleic acid sequence to which the
HPBF binds.

10   **BRIEF DESCRIPTION OF THE DRAWINGS**

Other advantages of the present invention will be readily appreciated as
the same becomes better understood by reference to the following detailed description
when considered in connection with the accompanying drawings wherein:

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RE 1 is a representation of a partial physical map of ERBB2 5'
region includin: .romoter area, where sev:~:! binding factors are indicated in black
boxes. The pr   which is the immediate :   ~noter region, spans - 22 to + 9
relative to the :   .nscription start site in th: .3B2 promoter.

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RE 2 presents the strategy u3ed to construct specific DNA-
sepharose resin :   :g double stranded oligonucleotide (probe B).

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention may be understood more readily by reference to
the following detailed description of specific embodiments and the Examples and
30   Figures included therein.

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According to the present invention, a purified and isolated DNA-binding factor which specifically binds to the promoter region of the ERBB2 gene sequence (Her-2/*neu* promoter binding factor: HPBF) has been found, as detailed in Examples 1-4 here below. (The factor has also been designated herein as ERBB2 promotor binding

5   protein: EPBP and as Tumor Enhancer Factor: TEF.) The factor was determined to be a protein as detailed in Example 5 below. The protein includes a peptide generated by asp-N digest with an N-terminal ten amino acid sequence of Aspartic Acid-Glycine-Aspartic acid-Asparagine-Phenylalanine-Proline-Leucine-Alanine-Proline-Phenylalanine (SEQ ID NO:1) as detailed in Example 8 here below. Further, the protein includes a

10   peptide generated by cyanogen bromide cleavage with an N-terminal ten amino acid sequence of Lysine- Isoleucine- Alanine- Isoleucine- Glutamic acid- Alanine- Glycine-Tyrosine- Aspartic acid- Phenylalanine (SEQ ID NO:2) as detailed in Example 8 here below.

15   The isolated protein has a molecular weight of about 44,000-47,000 daltons as measured by SDS-PAGE. Further the protein binds specifically to a double stranded-DNA (ds-DNA) probe of sense and anti-sense oligonucleotides having the sense sequence:

       5'----T A C - G A A T G A A G T T G T G A A G C T G A G A T T C C C C T C

20   C---3' (SEQ ID NO:3) and the anti-sense sequence

       3'-----------C T T A C T T C A A C A C T T C G A C T C T A A G G G G A G G -

       C A T---5' (SEQ ID NO:4), as detailed in Example 7 below. Microinjection into NIH-3T3 cells of the purified protein causes the induction of DNA synthesis in quiescent NIH-3T3 cells, as detailed in Example 9 below.

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       The DNA-binding protein (HPBF) is purified and isolated from tumor tissues using a ds-DNA probe of sense and anti-sense oligonucleotides having the sense sequence:

       5'----T A C - G A A T G A A G T T G T G A A G C T G A G A T T C C C C T C

30   C---3' (SEQ ID NO:3) and the anti-sense sequence

       3'-----------C T T A C T T C A A C A C T T C G A C T C T A A G G G G A G G -

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C A T---5' (SEQ ID NO:4) as more fully detailed in Example 6.

This DNA-binding protein has been detected at high concentrations in samples of adenocarcinoma-admixed with carcinoma *in situ* of the breast, whereas the
5   apparently benign breast tissue from the same quadrant area shows very minimal (almost unidentifiable) presence of this protein, and has also been found in the sera of patients with breast cancer, as detailed in Examples 2, 3 and 10. These studies indicate that this DNA-binding protein is specifically interacting with the promoter region of the ERBB2 gene during the transition of normal epithelial cells towards carcinoma *in situ* and
10   subsequently to the development of invasive breast carcinoma and the protein is soluble and excreted into the serum. The protein, therefore, provides an earlier indication of transition to a cancerous state than the gene product of the ERBB2 gene itself.

The present invention also provides an antibody that is specifically
15   reactive with HPBF. "Specifically reactive," as used herein describes an antibody or other ligand that specifically binds the HPBF protein and does not crossreact substantially with any antigen other than the HPBF protein. Antibody can include antibody fragments such as Fab fragments which retain the binding activity.

20   The antibody can be bound to a solid support substrate or conjugated with a detectable moiety or therapeutic compound or both bound and conjugated. Such conjugation techniques are well known in the art. For example, conjugation of fluorescent or enzymatic moieties can be performed as described in Johnstone & Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.
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The binding of antibodies to a solid support substrate is also well known in the art. (*See*, for example, Harlow and Lane, *Antibodies; A Laboratory Manual*, Cold Spring, Harbor Laboratory, Cold Spring Harbor, New York, 1988). The detectable moieties contemplated with the present invention can include fluorescent,
30   enzymatic and radioactive markers. Therapeutic drugs contemplated with the present invention can include cytotoxic moieties such as ricin A chain, diphtheria toxin and

16

detectable moieties contemplated with the present invention can include fluorescent, enzymatic and radioactive markers. Therapeutic drugs contemplated with the present invention can include cytotoxic moieties such as ricin A chain, diphtheria toxin and chemotherapeutic compounds. Such therapeutic drugs can be utilized for killing cancer cells expressing HPBF.

Immunoassays

Immunoassays such as immunofluorescence assays, radioimmunoassays (RIA), immunoblotting and enzyme linked immunosorbent assays (ELISA) can be readily adapted to accomplish the detection of HBPF. In general, ELISAs are the preferred immunoassays employed to assess the amount of HBPF in a specimen. Both polyclonal and monoclonal antibodies can be used in the assays. An ELISA method effective for the detection of HBPF protein can, for example, be as follows: (1) bind the antibody to a substrate; (2) contact the bound antibody with a fluid or tissue sample containing the antigen; (3) contact the above with secondary antibody bound to a detectable moiety (e.g., horseradish peroxidase enzyme or alkaline phosphatase enzyme); (4) contact the above with the substrate for the enzyme; (5) contact the above with a color reagent; and (6) observe color change. Available immunoassays are extensively described in the patent scientific literature. See, for example, United States Patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876.

Bioassays for Determining the Amount of
HPBF in a Biological Sample

The present invention provides a method of determining the amount of HPBF in a biological sample comprising the steps of contacting the biological sample with a substance which binds HPBF under conditions such that a complex between HPBF and the substance can be formed and determining the amount of the complex, the amount of complex indicating the amount of HPBF in the sample.

As contemplated herein, a biological sample includes any body fluid

17

which would contain the HPBF protein, such as blood, plasma, serum, and urineor any
cell containing the HPBF protein. Examples of cells include tissues taken from surgical
biopsies or isolated from a body fluid.

5   One example of the method of determining the amount of HPBF in a
biological sample is performed by contacting the biological sample with a nucleic acid
which binds HPBF under conditions to form a complex and determining the amount of
HPBF/nucleic acid complex, the amount of the complex indicates the amount of HPBF
in the sample. Nucleic acid sequences which bind HPBF to form a complex can be
10   identified as described herein in the Examples. For example, the nucleic acid sequence
of SEQ ID NO:3 binds HPBF as described herein.

          Determination of the amount of HPBF/nucleic acid complex can be
accomplished through techniques standard in the art. For example, the complex may be
15   precipitated out of a solution or detected by the addition of a detectable moiety
conjugated to the nucleic acid, as described, for example in Sambrook *et al.*, *Molecular
Cloning, A Laboratory Manual*, Cold Springs Harbor, New York, 1989).

          Another example of the method of determining the amount of HPBF in a
20   biological sample is performed by contacting the biological sample with an antibody
against HPBF under conditions such that a specific complex of an antibody and HPBF
can be formed and determining the amount of HPBF/antibody complex, the amount of
the complex indicating the amount of HPBF in the biological sample. Antibodies which
bind HPBF can be either monoclonal or polyclonal antibodies and can be obtained as
25   described herein in the Examples. Determination of HPBF/antibody complexes can be
accomplished using the immunoassays as described herein in the Examples.

          The present invention also provides a method of detecting the presence
of a cancer in a subject comprising determining the presence of a detectable amount of
30   HPBF in a biopsy from the subject, the presence of a detectable amount of HPBF,
relative to the absence of HPBF in a normal control, indicating the presence of a cancer.

18

The method of determining the presence of a detectable amount of HPBF in a biopsy

from the subject comprises the methods of determining the amount of HPBF in a

biological sample as described herein in the Examples. As used herein, "biopsy" means

any body fluids or cells which may contain HPBF which have been removed from the

5   subject suspected of having a cancer. Also, as used herein, "detectable amount" means

any amount of HPBF which is detectable by the methods of detection of HPBF

described herein, as compared to the absence of a detectable amount of HPBF in a

normal control biopsy taken from the same subject. When a normal biopsy sample and a

suspected cancerous biopsy sample are removed from the same subject, any amount of

10   HPBF present in the suspected sample, in greater quantities than an amount of HPBF

detected in a normal sample, is considered a detectable amount. A detectable amount of

HPBF is indicative of the presence of cancer, based on results of numerous studies as

cited herein.

15   The present invention further provides a method of determining the

prognosis of a subject having cancer comprising determining the presence of a

detectable amount of HPBF in a biopsy from the subject, the presence of a detectable

amount of HPBF, relative to the absence of HPBF in a normal control indicating a

decreased chance of long-term survival. A detectable amount of HPBF is indicative of

20   decreased chance of long-term survival based on the statistical correlations as described

herein.

Isolation of DNA Encoding HPBF

               The present invention provides an isolated nucleic acid encoding HPBF.

25   By "isolated" is meant separated from other nucleic acids found in humans. The nucleic

acid encoding HPBF is specific for humans expressing HPBF. By "specific" is meant an

isolated sequence which does not hybridize with other nucleic acids to prevent an

adequate hybridization with the nucleic acid encoding HPBF.

30   The isolated nucleic acid encoding HPBF can be obtained by standard

methods well known in the art. For example, a library of cDNA clones can be generated

19

and expressed in *E. coli* bacteria. Specific clones expressing HPBF or fragments thereof can be screened on colony blots using antibodies against HPBF generated as described in the Examples herein. Positive clones can then be sequenced by standard methods and the entire genes sequence of HPBF can be determined. (See, Sambrook *et al.*,

5   *Molecular Cloning, A Laboratory Manual*, Cold Springs Harbor, New York, 1989).

Also provided is an isolated nucleic acid that selectively hybridizes with the nucleic acid encoding HPBF under stringent conditions and has at least 70% and more preferably 80% and 90% complementarity with the segment and strand of the

10   nucleic acid of HPBF to which it hybridizes. As used herein to describe nucleic acids the term "selectively hybridizes" excludes the occasional randomly hybridizing nucleic acids as well as nucleic acids that encode other known promoter binding factors. Because the HPBF-encoding nucleic acid is double stranded, the selectively hybridizing nucleic acid can hybridize with either strand when the two strands of the coding

15   sequence are not hybridized to each other. The selectively hybridizing nucleic acids can be used, for example, as probes or primers for detecting the presence of a sample that has a nucleic acid to which it hybridizes. Alternatively, the nucleic acid can encode a segment of the HPBF protein. The conditions of hybridization are stringent, but may vary depending on the length of the nucleic acids.

20

Modifications to the nucleic acids of the invention are also contemplated as long as the essential structure and function of the polypeptide encoded by the nucleic acids are maintained. Likewise, fragments used as primers or probes can have substitutions as long as enough complementary bases exist for selective hybridization

25   (Kunkel *et al., Methods Enzymol.*, 154:367 (1987)).

Bioassays

The present invention provides a bioassay for screening substances for their ability to inhibit the activity of HPBF. Briefly, this can be accomplished by

30   cotransfection assays whereby a plasmid containing a promoter gene, such as the bacterial chloramphenicolacetyltransferase (CAT) gene, cloned directly downstream of

20

the ERBB2 promoter, can be cotransfected into a cultured cell line, such as COS7 cells,
with a second plasmid which has a promoter known to be active in the cultured cells,
cloned directly upstream of the HPBF gene. In such an assay, the HPBF gene encoding
the HPBF transcript will be transcribing HPBF messenger RNA which will then be

5   translated into HPBF protein. The HPBF protein then will be activating transcription of
the reporter gene through its interaction with the ERBB2 promoter. The products of
the reporter gene transcripts can then be quantitated. Such techniques for cotransfection
and detection of CAT gene products in cultured cell lines are very well known in the
art[98-101]. A cotransfected cell culture can then be contacted with compounds to screen

10   them for the ability to inhibit the activity of HPBF. A compound which inhibits the
activity of HPBF will inhibit the interaction of HPBF with the ERBB2 promoter. This
decreased interaction is quantifiable by monitoring the CAT enzyme produced as a result
of transcription directed by the ERBB2 promoter.

15   The present invention also provides a bioassay for screening substances
for the ability to inhibit the mitogenic activity of HPBF in cultured NIH3T3 cells.
NIH3T3 cells are highly sensitive to sarcoma virus formation and HPBF is known to
produce mitogenic effect when introduced into these cells[102,103]. Briefly, quiescent
NIH3T3 cultured cells are microinjected with HPBF and observed for any mitogenic

20   effect, such as the formation of morphologically recognizable foci (cells no longer
growing in an organized manner and as a monolayer, but contact inhibited and
disorganized, eventually growing in disorganized multiple layers). Alternatively, DNA
synthesis levels can be monitored both pre and post-injection as a direct measure of
changes in genome replication[103].

25

               Using this mitogenic assay, one can screen substances for their ability to
inhibit the known mitogenic activity of HPBF. Such substances can be co-injected into
quiescent NIH3T3 cells with HPBF and the mitogenic activity can then be compared to
the mitogenic activity of HPBF or such substance injected alone. One can then readily

30   determine whether a substance has an inhibitory effect on the mitogenic activity of
HPBF.

21

Inhibition of Biological Activity of HPBF

The present invention provides a method of inhibiting a biological activity
mediated by HPBF comprising preventing the HPBF from binding to the promoter
region of the ERBB2 gene sequence.

5

In one example, the present invention provides a method of inhibiting a
biological activity mediated by HPBF comprising preventing the HPBF from binding to
the promoter region of the ERBB2 gene sequence wherein the binding to the promoter
region is prevented by an antisense nucleotide sequence. The antisense oligonucleotide

10   can be generated using well known nucleic acid synthesis methods as demonstrated in
the Examples.

In another example, the present invention provides a method of inhibiting
a biological activity mediated by HPBF comprising preventing the HPBF from binding

15   to the promoter region of the ERBB2 gene sequence wherein the binding to the
promoter region is prevented by a nongenomic nucleic acid sequence to which the
HPBF binds.

A method to inhibit a biological activity of HPBF and decrease ERBB2

20   activity can use antisense or triplex oligonucleotide analogues or expression constructs.
This entails introducing into the cell a nucleic acid sufficiently complementary in
sequence so as to selectively hybridize to the target gene or message. Triplex inhibition
relies on the transcriptional inhibition of the target gene and can be extremely efficient
since only a few copies per cell are required to achieve complete inhibition. Antisense

25   methodology on the other hand inhibits the normal processing, translation or half-life of
the target message. Such methods are well known to one skilled in the art.

Although longer sequences can be used to achieve inhibition, antisense
and triplex methods generally involve the treatment of cells or tissues with a relatively

30   short oligonucleotide. The oligonucleotide can be either deoxyribo- or ribonucleic acid
and must be of sufficient length to form a stable duplex or triplex with the target RNA

22

or DNA at physiological temperatures and salt concentrations. It should also be of sufficient complementarity selectively hybridize to the target nucleic acid. Oligonucleotide lengths sufficient to achieve this specificity are generally about 12 to 60 nucleotides long, preferably about 18 to 32 nucleotides long. In addition to length,

5   hybridization specificity is also influenced by GC content and primary sequence of the oligonucleotide. Such principles are well known in the art and can be routinely determined by one who is skilled in the art.

       The composition of the antisense or triplex oligonucleotides can also

10   influence the efficiency of inhibition. For example, it is preferable to use oligonucleotides that are resistant to degradation by the action of endogenous nucleases. Nuclease resistance will confer a longer *in vivo* half-life onto the oligonucleotide and therefore increase its efficacy by reducing the required dose. Greater efficacy can also be obtained by modifying the oligonucleotide so that it is more permeable to cell

15   membranes. Such modifications are well known in the art and include the alteration of the negatively charged phosphate backbone of the oligonucleotide to uncharged atoms such as sulfur and carbon. Specific examples of such modifications include oligonucleotides that contain methylphosphonate and thiophosphonate moieties in place of phosphate. These modified oligonucleotides can be applied directly to the cells or

20   tissues to achieve entry into the cells and inhibition of HPBf activity. Other types of modifications exist as well and are known to one skilled in the art.

       Recombinant methods known in the art can also be used to achieve the antisense or triplex inhibition of a target nucleic acid. For example, vectors containing

25   antisense nucleic acids can be employed to express protein or antisense message to reduce the expression of the target nucleic acid and therefore its activity. Such vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the antisense or triplex sequences. Other beneficial characteristics can also be contained within the

30   vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used

23

either as plasmids or as bacteriophage vectors. Examples of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill

5   in the art will know which host systems are compatible with a particular vector.

The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found described in Sambrook *et al., Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New

10   York (1992), in Ausubel *et al., Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989), and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. Introduction of nucleic acids by infection offers several advantages over the other listed methods. Higher efficiency can be obtained due to their infectious nature. Moreover,

15   viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the antisense vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

20

A specific example of a DNA viral vector for introducing and expressing antisense nucleic acids is the adenovirus derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired recombinant sequences such as antisense

25   sequences. This vector can be used to infect cells that have an adenovirus receptor which includes most cancers of epithelial origin as well as others. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells can include, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

30

Additional features can be added to the vector to ensure its safety and/or

24

enhance its therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the antibiotic gancyclovir. Negative selection is therefore a means by

5   which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or antisense sequence, cellular transformation will not occur. Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are

10   specific for the desired cell type.

Recombinant viral vectors are another example of vectors useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for

15   example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which were not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are

20   unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses

25   infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. The vector to be used in the methods of the invention will depend on desired cell type to be targeted. For example, if breast cancer is to be treated by decreasing the HPBF activity of cells affected by the

30   disease, then a vector specific for such epithelial cells should be used. Likewise, if diseases or pathological conditions of the hematopoietic system are to be treated, then a

viral vector that is specific for blood cells and their precursors, preferably for the specific type of hematopoietic cell, should be used.

Retroviral vectors can be constructed to function either as infectious

5   particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also

10   engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed though subsequent rounds of infection. The specific type of vector will

15   depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

HPBF antisense-encoding viral vectors can be administered in several

20   ways to obtain expression and therefore decrease the activity of HPBF in cells affected by the disease or pathological condition. If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration can provide a quicker and more effective treatment, administration can also be performed

25   by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into the spinal fluid can also be used as a mode of administration, especially in the case of neuro-degenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

30

An alternate mode of administration of HPBF antisense-encoding vectors

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can be by direct inoculation locally at the site of the disease or pathological condition or by inoculation into the vascular system supplying the tumor with nutrients. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve HPBF expression in a majority of the targeted cells.

5   Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area. If expression is desired in only a specific subset of cells within the inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be, for

10   example, viral vectors, viral genome, plasmids, phagemids and the like. Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

15   In addition to the antisense methods described above, other methods can be used as well to decrease the activity of HPBF and achieve the down regulation of ERBB2 activity. For example, oligonucleotides which compete for the HPBF binding site within the ERBb2 regulatory elements can be used to competitively inhibit HPBF binding to ERBB2. Such oligonucleotides can be, for example, methylphosphonates and

20   thiophosphonates which permeate the cell membrane. Alternatively, vectors which express such sequences or contain the HPBF binding element can also be used to achieve the same result as the oligonucleotides. Modes of administration for the competitive inhibition are similar to that described above for the antisense vectors and oligonucleotides.

25

        The present invention also provides for a bioassay for screening substances for the ability to inhibit the production of HPBF comprising administering the substance to a cell having a gene activity expressing the HPBF gene (an activated gene encoding HPBF) and then determining the amount of HPBF subsequently produced.

30

        Stabely transformed cell lines expressing HPBF can be constructed in

27

several ways. One example of such a technique is integrating genetic material known to encode HPBF into the chromosome of a host cell. Such integration, usually mediated through transfection of the DNA by DEAE Dextran, Calcium Phosphate precipitation, or via liposome encapsulation, can be coupled to the introduction of genes utilized to

5   enhance gene expression. For example, the metabolic inhibitor, dihydrofolate reductase can be selected as the cotransfecting DNA to achieve DNA amplification and therefore enhanced or activated gene expression. In such a system, co-transfected cells are treated with methotrexate, a known inhibitor of dihydrofolate reductase. Cells resistant to methotrexate obtain this resistance by amplifying the numbers of dihydrofolate

10   reductase genes. Genes other than the dihydrofolate gene are amplified as well [104].

Amplification of the cotransfected gene can be verified in several ways. These techniques can be, but are not limited to quantitative polymerase chain reaction, Southern blot hybridization, and dot blot hybridization. The presence of enhanced levels

15   of HPBF protein can also be detected. One example of such a technique is through separating cellular proteins by polyacrylamide gel electrophoresis, either single or two dimensional, and then visualized by staining, or through antigen-antibody interaction. Such techniques are very well known in the art (Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Springs Harbor, New York, 1989).

20

Cells expressing HPBF can then be contacted with substances to screen for those which decrease the amount of HPBF produced. Techniques for detecting a change in the amount of HPBF produced can be, but are not limited to polyacrylamide gel electrophoresis, enzyme linked immunosorbent assay and by bioassay.

25

The invention will now be demonstrated by the following non-restrictive examples:

The present invention is more particularly described in the following

30   examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

28

EXAMPLES

GENERAL METHODS

<u>Preparation of Cytoplasmic and Nuclear Extracts</u>

      The cytoplasmic and nuclear extracts from tissues and cells were

5   prepared following standard procedures.[92] Briefly, cells were trypsinized (1×10^9) and

centrifuged at 5,500 rpm for 10 minutes. The supernatant was discarded and the pellet

washed twice in 5x volume of phosphate buffered saline (PBS). Centrifugation step was

repeated. The cell pellet was resuspended in 5x pellet volume of ice-cold buffer A

(15mM KCl, 10mM Hepes, 2mM $MgCl_2$, 0.1mM EDTA). All remaining steps were

10   performed at 4°C. The cells and tissues were homogenized using a glass-glass dounce

homogenizer. The homogenization was complete when >85% of the cells were lysed as

determined by phase contrast microscopy. The homogenate was mixed with 1/10 vol of

buffer B (1M KCl, 50mM Hepes, 30mM $MgCl_2$, 0.1mM EDTA, 1mM DTT) and left on

ice for 4–5 minutes followed by centrifugation at 10,000 rpm for 10 minutes. The

15   supernatant was reserved for cytoplasmic extraction. The nuclear pellet was

resuspended in 5 ml in a buffer of 9 parts buffer A and 1 part buffer B. Ammonium

sulphate (4M, pH 7.9) was added to the extract to a final concentration of 0.36M and

the nuclear proteins were extracted by gentle rocking on a shaker at 4°C for 30 minutes.

The DNA was separated from the proteins by centrifugation of the lysate at 150,000g

20   for 60 minutes. The supernatant was collected and the proteins were precipitated by the

addition of 0.25 g ammonium sulphate per ml of supernatant. The precipitated proteins

were collected by centrifugation at 150,000g for 15 minutes and suspended in one-half

of the original cell pellet volume in buffer C (10% Glycerol, 25mM Hepes (pH 7.6),

40mM KCl, 0.1mM EDTA, 1mM DTT). The proteins were dialyzed against Buffer C

25   for 2–4 hours, collected in a tube and centrifuged at 10,000 rpm for 10 minutes. Protein

concentration was determined by Bio-Rad® protein reagents and the extract was stored

in smaller aliquots at -70°C.

      For cytoplasmic extraction of the reserved supernatant, 5 g of ammonium

30   sulfate was added per 10 ml of supernatant and dissolved by gentle shaking at 4°C. The

supernatant was then centrifuged the same way as for nuclear extract preparation. The

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precipitate was suspended in Buffer C and dialyzed against Buffer C as for nuclear
extract preparation.

Preparation of Double Stranded Oligonucleotides

5   An aliquot of equal moles of sense and anti-sense oligonucleotides in
H_2O was mixed and the mixture was incubated sequentially at 95-100°C for 10
minutes, at 65°C for 1 hour, 37°C for 2-3 hours and at RT for 5 hours to form the
double stranded (ds) oligonucleotides. The DNA was precipitated by the addition of
0.3M NaOAC and 2.5 vol of 100% ETOH. The precipitated DNA was collected by
10   centrifugation and washed once with 70% ETOH and the pellet was dried under
vacuum. The DNA was suspended in H_2O and the exact concentration is determined by
spectrophotometry.

5' End Labelling of Double Stranded Oligonucleotides

15   The 5' end labelling was accomplished essentially according to the
manufacturer's protocol (Stratagene) using α-^{32}P-ATP and the probe was purified
through gel extraction. The labeled oligonucleotide was separated through an 8-10%
PAGE in 1x TBE (Tris-borate-EDTA buffer). Loading of the samples was done by
mixing with 5x dye.[93] Electrophoresis was continued at 30-36 mA for about 2-4 hours
20   and the gel was exposed to Kodak® XAR-5 film and developed after about 10 minutes
of exposure. The ds oligonucleotide band was cut from the gel, cut into smaller pieces
and mixed with two volumes of a mixture containing 0.5M NH_4OAC and 1mM EDTA
and allowed to shake at 37°C overnight. The whole suspension was passed through
glass wool in a 3 ml syringe and the clear radioactively labeled DNA solution was
25   collected. Yeast tRNA, to a final concentration of 30-40 μg/ml, was added to the
labelled DNA and precipitated with 2.5 volume of ETOH overnight at -20°C. The tube
was then centrifuged, the pellet washed once with 70% ETOH, and vacuum dried. The
vacuum dried pellet was suspended in TE and the radioactivity was determined by
counting an aliquot.

30

30

Gel Mobility Shift Assay (GMSA)

The tissue or cell extract was mixed with 5x binding buffer (125 mM HEPES, pH 7, containing 50 mM KCl, 5 mM DTT, 5 mM EDTA, 50% Glycerol and 0.25% NP-40), poly dI:dC (1-2 μg) and H_2O, and the mixture incubated at RT for 10 minutes in a reaction volume of 20-25 μl. The labelled probe (12,000-15,000 cpm) was then added to the mixture and the reaction was continued at RT for 40 minutes. At the end of the reaction time, 1 μl of 5x dye was added and loaded on a 6% pre-run PAGE in 1x TBE. The electrophoresis was continued at 32-36 mAmp. The gel was dried and exposed to the X-ray film.

Southwestern (DNA-Protein) Blot Assay

For the Southwestern procedure, the cytoplasmic or nuclear proteins were separated on SDS-PAGE (10% separating gel)[93] under reducing conditions and the proteins were electrotransferred onto nylon membrane (Immobilon® P membrane). The membrane was washed three times (one hour each) with renaturation buffer (10mM Tris-Hcl, pH 7.5, 150mM NaCl, 10mM DTT, 2.5% NP-40, 10% Glycerol and 5% nonfat dry milk) and rinsed briefly in binding buffer (10mM Tris-Hcl, pH 7.5, 40mM NaCl, 1mM DTT, 1mM EDTA, 8% Glycerol and 0.125% non-fat dry milk). The membrane was then incubated in 15 ml of binding buffer plus 45 μg poly (dI-dC), 5mM $MgCl_2$ and 1 x 10^6 cpm of ^{32}P-labelled DNA probe per ml for 15 hours at RT with continuous agitation. The membrane was washed four times (30 minutes each) in 10mM Tris-Hcl, pH 7.5 containing 50mM NaCl and exposed to X-ray film.

Preparation of Sequence-Specific DNA-Sepharose Resin

Chemically synthesized complementary oligonucleotides corresponding to -22 to +9 sequences (see Examples) of ERBB2 were annealed, 5'-phosphorylated, ligated and coupled to CNBr-activated sepharose 4B essentially according to the method of Kadonaga and Tjian.[94]

Affinity Purification of Sequence-Specific DNA-binding Protein

All operations were performed at 4°C. The oligonucleotide-affinity resin

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(1 ml) was equilibrated with buffer Z (0.1 M KCl, 25 mM HEPES pH 7.6, 12.5 mM MgCl$_2$, 15% glycerol, 1 mM DTT and 0.05% NP-40). Cytoplasmic and/or nuclear extracts (10 ml) were dialyzed against buffer Z, combined with 250 μg of salmon sperm DNA and allowed to stand for 10 minutes on ice. This protein-DNA mixture was then

5   mixed with the ERBB2-sepharose resin for 5-8 hours at 4°C with occasional shaking and then loaded onto a column. The mixture was allowed to elute under gravity flow and washed with 4 to 5 column bed volumes of buffer Z. At this stage, the column was stopped, buffer Z containing 1M KCl (10 ml) was added and mixed with the resin thoroughly. The resin was allowed to stand for 15 minutes with occasional mixing and

10   then the protein was eluted. This first cycle higher salt eluate was diluted in 0.1 M KCl buffer Z, mixed with salmon sperm DNA and the whole procedure was repeated for second cycle purification identical to the first cycle.

Cell Lines and Primary Tumor Tissue

15   Cell lines NIH-3T3, (ATCC Accession No. CRL 1658) and SKBR3 (ATCC Accession No. HTB 30) were used. Primary breast cancer samples were obtained from mastectomy specimens. Pathology of each sample was confirmed using H&E stained frozen as well as formalin fixed tissue sections.

20   EXAMPLE 1

Preparation of Probes

            In order to identify specific factor(s) that are responsible for the regulation of the ERBB2 gene, three sets of sense and anti-sense ds-oligonucleotides based on the DNA sequence of a genomic clone of the ERBB2

25   promoter region entered in the Genbank were prepared. The promoter DNA sequence was analyzed through a Genbank data search.[21] The Genbank Accession numbers were M16789[95] and M16892[96]. The DNA sequences of these three sets of oligonucleotides are indicated below and a map is shown in Figure 1.

30   The first sets were from base -79 to +9, relative to the last transcription start site (+1). The last transcription start site is located at position -178 relative to the

32

first translational start codon "ATG". Therefore, the first set of oligonucleotides are from -258 to -169 relative to the first translational start codon "ATG". Position -178 is located at 21 bp downstream from the last TATAA box (-204 to -200 relative to the translational start codon). This set (Set 1, Probe C) of oligonucleotides consists of

5   DNA sequences from the transcriptional start site, including TATAA and CAAT boxes. The second set (Set 2, Probe A) was from the same region, excluding TATAA and CAAT boxes (-79 to -22 relative to the transcriptional start site). The third set (Set 3, Probe B) of oligonucleotides was also from the same region excluding TATAA and CAAT boxes, but including transcriptional start site (-22 to +9), and including

10   immediate base sequences upstream from the transcriptional start site, plus a few bases downstream of the transcriptional start site.

Set No. 1 to create probe C:

Sense Sequence: contains a three nucleotide 5' overhang.

15   5'---GCT-CCCAATCACAGGAGAAGGAGGAGGTGGAGGA
GGAGGGCTGCTTGAGGAAGTATAAGAATGAAGTTGTG
AAGCTGAGATTCCCCTCC---3'(SEQ ID NO:5)

Antisense Sequence: contains a three nucleotide 5' overhang.

20   3'---------GGGTTAGTGTCCTCTTCCTCCTCCACCTCCTCC
TCCCGACGAACTCCTTCATATTCTTACTTCAACACTTC
GACTCTAAGGGGAGG-CAT---5' (SEQ ID NO:6)

Set No. 2 to create probe A:

25   Sense Sequence: contains a three nucleotide 5' overhang.

5'---GCT-CCCAATCACAGGAGAAGGAGGAGGTGGAGGA
GGAGGGCTGCTTG
AGGAAGTATAAGA---3' (SEQ ID NO:7)

30   Antisense Sequence: contains a three nucleotide 5' overhang.

3'---------GGGTTAGTGTCCTCTTCCTCCTCCACCTCCTCC

33

T C C C G A C G A A C T C C T T C A T A T T C T- C A T---5' (SEQ ID NO:8)

Set No. 3 to create probe B :

Sense Sequence: contains a three nucleotide 5' overhang.

5   5'---T A C -G A A T G A A G T T G T G A A G C T G A G A T T C C C C T C

C---3' (SEQ ID NO:3)

Antisense Sequence: contains a three nucleotide 5' overhang.

3'------------C T T A C T T C A A C A C T T C G A C T C T A A G G G G A G G-

10   C A T---5' (SEQ ID NO:4).

The sequence and location of probe B is indicated in Figure 1. The

position for SP1 binding sites and the classical CAAT and TATAA box is also indicated.

All three sets of these oligonucleotide were used to generate double stranded DNA

15   (ds-oligonucleotide).

EXAMPLE 2

Analysis by GMSA

Radioisotopically (^{32}P) labelled ds-oligonucleotide probes were made and

20   Gel Mobility Shift Assays (GMSA) were carried out. For initial experiments, nuclear

and cytoplasmic extracts were made from a benign specimen (normal) and a paired

specimen of benign and tumor (adenocarcinoma admixed with carcinoma *in situ*), freshly

collected from breast mastectomies, as well as SKRB3 cell extracts.

25   Nuclear and cytoplasmic extracts from a benign specimen and from a

paired specimen of benign and tumor (pathologically diagnosed as adenocarcinoma)

from the breast were analyzed by GMSA using all three probes. Probe B identified a

specific factor which is present only in the nuclear and cytoplasmic extract of the tumor

sample. The presence of this factor was totally absent in the nuclear extracts of benign

30   tissue. However, the cytoplasmic extracts of both of the benign tissue samples show the

presence of this factor at an extremely low level.

34

EXAMPLE 3

Further GMSA Analysis with Probe B

A series of four breast specimens of paired benign (B) and tumor (T) was analyzed similarly using GMSA and utilizing Probe B. The benign and tumor tissues

5   were taken from the same quadrant area of the excised tissue. The histopathology examination identified the apparently benign area for use in the assay. Nuclear and cytoplasmic extracts from an atypical hyperplastic breast specimen were included.

These results clearly show the presence of a probe-B-specific binding

10   factor in the tumor extracts of both nuclei and cytoplasm. The nuclear extracts of the apparently benign tissue from the same quadrant was completely devoid of this factor in this assay system. However, the cytoplasmic extracts of apparently benign and atypical hyperplastic tissue show the presence of this binding factor at a low level. It is not clear if the histopathologically apparently benign tissue from the same quadrant as the tumor

15   is truly benign or whether it is in an early pre-cancerous stage which this assay recognizes. Similarly, HPBF has also been detected from cytoplasmic/nuclear extracts of a breast cancer cell line (SKBR3) known to overexpress ERBB2.

EXAMPLE 4

20   ### Binding Specificity of Factor

The binding specificity of the factor was confirmed with a sample which showed highest binding with probe B. Nuclear extracts of benign tissue were negative, whereas nuclear and cytoplasmic extracts of tumor specimens were positive for the Probe-B-binding factor. Binding of this factor with Probe B was completely abolished

25   by excess unlabelled Probe B. This binding was not abolished using 50 fold unlabelled NFkB or SP1 probe, indicating that the binding of this factor is Probe-B-specific.

EXAMPLE 5

Determination of Factor as Protein

30   It was next determined that the binding factor (HPBF) is a protein.
For this, the nuclear and cytoplasmic extracts were fractionated through

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SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to nylon membrane and reacted with ^{32}P-labelled probe B (Southwestern assay). Both the membranes show binding activity with probe B and probe A.

5   A protein of about 50 kDa can bind to probe B only with tumor cell extracts (nuclear and cytoplasmic). The nuclear and cytoplasmic extracts of benign tissue failed to show any signal in the Southwestern assay, indicating that the level of this DNA-binding protein is extremely low in apparently benign breast tissue.

10   EXAMPLE 6

Isolation and Purification of HPBF

In order to isolate and purify the probe-B-specific DNA-binding protein (HPBF), a strategy for the purification of DNA-binding protein was used. This strategy is diagramed in Figure 2, using ds-oligonucleotide probe B to generate an

15   affinity resin.

Pooled cytoplasmic extract from three breast tumor specimens were subjected to the affinity purification. The extracts were passed through the affinity column and washed. The bound proteins were eluted with high salt buffer and three one

20   milliliter fractions were collected. The proteins in the high salt eluate were fractionated through SDS-PAGE and silver-stained. The high salt wash in three fractions showed a specific protein at a very high concentration at around 44,000-47,000 dalton molecular weight. This again demonstrates the presence of a major protein, HPBF, of about 50 kDa as has been previously shown in the Southwestern assay. HPBF was dialyzed

25   against GMSA binding buffer and stored in aliquots at -70°C.

EXAMPLE 7

Binding Specificity of Purified HPBF

The binding specificity of the purified HPBF was tested using GMSA

30   and labelled probe B.

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Only the tumor extract and purified HPBF bound DNA and formed a complex with probe B. The probe-B-specific binding protein is present in the tumor tissue specimen and the affinity purified protein. The benign extract did not show any binding. The specificity of the binding was competed out by unlabelled probe B,

5   whereas a non-specific probe was unable to compete for the binding activity.

These results clearly document the identification of a protein factor (a DNA-binding protein), HPBF, which specifically binds to the promoter region of the ERBB2 gene sequences.

10

EXAMPLE 8

Amino Acid Sequence of Peptide of HPBF

An asp-N digest of the purified protein was performed following routine procedures well known to those skilled in the art. An N-terminal ten amino acid

15   sequence of a peptide generated by the asp-N digest was determined using an automated protein micro sequencer. The ten amino acid sequence was determined to be Aspartic acid- Glycine- Aspartic acid- Asparagine- Phenylalanine- Proline- Leucine- Alanine- Proline- Phenylalanine (D G D N F P L A P F) (SEQ ID NO:1). It should be noted that the amino acid sequence of the protein may be slightly different due to possible

20   sequencing errors. Such errors can be determined by repeating the methods to confirm sequence accuracy. The sequence was compared with known amino acid sequences in Genbank and no matches were found, indicating the novel nature of this peptide.

Further, a cyanogen bromide cleavage of the purified protein was

25   performed following routine procedures well known to those skilled in the art. An N-terminal ten amino acid sequence of a peptide generated by the cyanogen bromide cleavage was determined using an automated protein micro sequencer. The ten amino acid sequence was determined to be Lysine- Isoleucine- Alanine- Isoleucine- Glutamic acid- Alanine- Glycine- Tyrosine- Aspartic acid- Phenylalanine (K I A I E A G Y D F)

30   (SEQ ID NO:2). The sequence was compared with known amino acid sequences in Genbank and no match was found, indicating the novel nature of this peptide.

37

Therefore, these results indicate that HPBF (ERBB2 gene specific DNA-binding protein) is a newly discovered protein with known biological function, that has never been documented.

5   EXAMPLE 9

HPBF Induces Cell Proliferation

Purified and isolated HPBF was micro-injected into serum-starved NIH-3T3 cells as has been described in the scientific literature.[97]

10   Microinjection of HPBF into the quiescent NIH 3T3 cells induced the onset of DNA synthesis as detailed in TABLE 1 herein. DNA synthesis increased 12–13 fold with HPBF. The DNA synthesis was increased 28 fold in the presence of the *Ras* oncogene and HPBF, suggesting that the factor either has a mitogenic activity or is a component of mitogenic signalling pathways. The *Ras* oncogene was microinjected at

15   an amount that gives minimal stimulation, as shown in Table I, since maximal stimulation as reported by Smith *et al.*[97] would not allow the HPBF response to be measured. Bovine serum albumin (BSA) was used as a control and showed, at most, a two-fold induction compared to the twelve to thirteen-fold increase induced by two separate extracts of HPBF. This induction of cell proliferation can be competed out

20   slightly by incubating with probe B (ds-oligonucleotide 3), but not with nonspecific probe A (ds-oligonucleotide 2).

38

TABLE I

Sample	% Injected Cells in S-Phase	Fold Induction
BSA	3	2 (1)
HPBF extract 1	38	13 (4)
HPBF extract 2	32	12 (3)
HPBF-1 + Probe A	25	9 (3)
HPBF-1 + Probe B	16	4 (2)
c-Ras	19	5 (2)
HPBF-1 + c-Ras	72	28 (7)

EXAMPLE 10

HPBF Can Be Measured in Sera

An ELISA assay of sera from breast, pancreas and kidney cancer patients against an anti-HPBF polyclonal antiserum demonstrated the presence of HPBF in the sera of breast cancer patients.

The polyclonal anti-HPBF sera were developed in hyperimmunized mice and were a pool of sera from three mice. The mice were being injected with purified and isolated HPBF for the production of monoclonal antibodies and the sera were obtained to determine the response of the immunized mice to the purified protein.

EXAMPLE 11

Production of Polyclonal and Monoclonal Antibodies

Polyclonal antibodies against the human breast tumor-derived protein (HPBF) found in both nucleus and cytoplasm, were prepared by immunization of a NZW rabbit. The material used for immunization was purified from a crude nuclear

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extract by oligonucleotide affinity chromatography. The animal was injected with the purified protein emulsified with Freund's Complete Aduvant for the initial injection, then emulsified with Freund's Incomplete Aduvant for a second injection, and finally boosted with an injection of protein antigen in aqueous phase only. The animal was bled at

5   weekly intervals and the serum analyzed for antibody activity using ELISA methodology with the purified antigen coated on the plate. The antiserum at peak development could be diluted >1:10,000 and still retain activity. Also, the antiserum was also used in a Western blot format to identify the antigen on a polyacrylamide gel at the correct molecular weight. This antibody retained activity after purification of the

10   immunoglobulin by protein A-sepharose chromatography.

Monoclonal antibodies specifically reactive with HPBF protein were also prepared by immunizing a Balb/cAnnCr mouse with the affinity-purified protein after a further purification by cutting the specific band from a polyacrylamide gel. A similar

15   immunization protocol was used, as described for polyclonal antibody production. After the mouse antiserum was shown to have antibody activity by ELISA testing, the animal was sacrificed and the spleen harvested. A spleen cell suspension was used to do a standard polyethylene glycol 1500 mediated-cell fusion with mouse myeloma 8.653 cells to form hybrids. Culture supernatants from the resulting cell hybridomas were screened

20   for antibody activity using the same ELISA method. Antibody positive wells were cloned in two stages by limiting dilution to derive the present twenty-one clones that are being evaluated. All have antibody activity in the ELISA, and some are Western blot positive as well. Purified antibody has been made from some of these clones, and some of these, as well as the polyclonal antibody react with breast cancer cells in

25   immunohistochemical studies.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of

30   words of description rather than of limitation.

40

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention

5   pertains.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent

10   that they are included in the accompanying claims.

Throughout this application various publications are referenced by full citation or numbers. Full citations for the publications referenced by number are listed below. The disclosures of these publications in their entireties are hereby incorporated

15   by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Raziuddin
              Sarkar, Fazlul H

    (ii) TITLE OF INVENTION: ERBB2 PROMOTER BINDING PROTEIN IN
                      NEOPLASTIC DISEASE

    (iii) NUMBER OF SEQUENCES: 15

    (iv) CORRESPONDENCE ADDRESS:
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        (F) ZIP: 30303-1811

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: David G. Perryman
        (B) REGISTRATION NUMBER: 33,438
        (C) REFERENCE/DOCKET NUMBER: 1414.608

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (404) 688-0770
        (B) TELEFAX: (404) 688-9880

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

    Asp Gly Asp Asn Phe Pro Leu Ala Pro Phe
    1   5   10

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

    Lys Ile Ala Ile Glu Ala Gly Tyr Asp Phe
    1   5   10

51

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TACGAATGAA GTTGTGAAGC TGAGATTCCC CTCC   34

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTTACTTCAA CACTTCGACT CTAAGGGGAG GCAT   34

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 89 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTCCCAATC ACAGGAGAAG GAGGAGGTGG AGGAGGAGGG CTGCTTGAGG AAGTATAAGA   60

ATGAAGTTGT GAAGCTGAGA TTCCCCTCC   89

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 89 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGTTAGTGT CCTCTTCCTC CTCCACCTCC TCCTCCCGAC GAACTCCTTC ATATTCTTAC   60

TTCAACACTT CGACTCTAAG GGGAGGCAT   89

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 60 base pairs
        (B) TYPE: nucleic acid

52

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            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCTCCCAATC ACAGGAGAAG GAGGAGGTGG AGGAGGAGGG CTGCTTGAGG AAGTATAAGA    60


(2) INFORMATION FOR SEQ ID NO:8:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 60 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGTTAGTGT CCTCTTCCTC CTCCACCTCC TCCTCCCGAC GAACTCCTTC ATATTCTCAT    60


(2) INFORMATION FOR SEQ ID NO:9:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 4530 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AATTCTCGAG CTCGTCGACC GGTCGACGAG CTCGAGGGTC GACGAGCTCG AGGGCGCGCG    60

CCCGGCCCCC ACCCCTCGCA GCACCCCGCG CCCCGCGCCC TCCCAGCCGG GTCCAGCCGG   120

AGCCATGGGG CCGGAGCCGC AGTGAGCACC ATGGAGCTGG CGGCCTTGTG CCGCTGGGGG   180

CTCCTCCTCG CCCTCTTGCC CCCCGGAGCC GCGAGCACCC AAGTGTGCAC CGGCACAGAC   240

ATGAAGCTGC GGCTCCCTGC CAGTCCCGAG ACCCACCTGG ACATGCTCCG CCACCTCTAC   300

CAGGGCTGCC AGGTGGTGCA GGGAAACCTG GAACTCACCT ACCTGCCCAC CAATGCCAGC   360

CTGTCCTTCC TGCAGGATAT CCAGGAGGTG CAGGGCTACG TGCTCATCGC TCACAACCAA   420

GTGAGGCAGG TCCCACTGCA GAGGCTGCGG ATTGTGCGAG GCACCCAGCT CTTTGAGGAC   480

AACTATGCCC TGGCCGTGCT AGACAATGGA GACCCGCTGA ACAATACCAC CCCTGTCACA   540

GGGGCCTCCC CAGGAGGCCT GCGGGAGCTG CAGCTTCGAA GCCTCACAGA GATCTTGAAA   600

GGAGGGGTCT TGATCCAGCG GAACCCCCAG CTCTGCTACC AGGACACGAT TTTGTGGAAG   660

GACATCTTCC ACAAGAACAA CCAGCTGGCT CTCACACTGA TAGACACCAA CCGCTCTCGG   720

GCCTGCCACC CCTGTTCTCC GATGTGTAAG GGCTCCCGCT GCTGGGGAGA GAGTTCTGAG   780

GATTGTCAGA GCCTGACGCG CACTGTCTGT GCCGGTGGCT GTGCCCGCTG CAAGGGGCCA   840

CTGCCCACTG ACTGCTGCCA TGAGCAGTGT GCTGCCGGCT GCACGGGCCC CAAGCACTCT   900

GACTGCCTGG CCTGCCTCCA CTTCAACCAC AGTGGCATCT GTGAGCTGCA CTGCCCAGCC   960
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53

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CTGGTCACCT ACAACACAGA CACGTTTGAG TCCATGCCCA ATCCCGAGGG CCGGTATACA 1020

TTCGGCGCCA GCTGTGTGAC TGCCTGTCCC TACAACTACC TTTCTACGGA CGTGGGATCC 1080

TGCACCCTCG TCTGCCCCCT GCACAACCAA GAGGTGACAG CAGAGGATGG AACACAGCGG 1140

TGTGAGAAGT GCAGCAAGCC CTGTGCCCGA GTGTGCTATG GTCTGGGCAT GGAGCACTTG 1200

CGAGAGGTGA GGGCAGTTAC CAGTGCCAAT ATCCAGGAGT TTGCTGGCTG CAAGAAGATC 1260

TTTGGGAGCC TGGCATTTCT GCCGGAGAGC TTTGATGGGG ACCCAGCCTC CAACACTGCC 1320

CCGCTCCAGC CAGAGCAGCT CCAAGTGTTT GAGACTCTGG AAGAGATCAC AGGTTACCTA 1380

TACATCTCAG CATGGCCGGA CAGCCTGCCT GACCTCAGCG TCTTCCAGAA CCTGCAAGTA 1440

ATCCGGGGAC GAATTCTGCA CAATGGCGCC TACTCGCTGA CCCTGCAAGG GCTGGGCATC 1500

AGCTGGCTGG GGCTGCGCTC ACTGAGGGAA CTGGGCAGTG GACTGGCCCT CATCCACCAT 1560

AACACCCACC TCTGCTTCGT GCACACGGTG CCCTGGGACC AGCTCTTTCG GAACCCGCAC 1620

CAAGCTCTGC TCCACACTGC CAACCGGCCA GAGGACGAGT GTGTGGGCGA GGGCCTGGCC 1680

TGCCACCAGC TGTGCGCCCG AGGGCACTGC TGGGGTCCAG GGCCCACCCA GTGTGTCAAC 1740

TGCAGCCAGT TCCTTCGGGG CCAGGAGTGC GTGGAGGAAT GCCGAGTACT GCAGGGGCTC 1800

CCCAGGGAGT ATGTGAATGC CAGGCACTGT TTGCCGTGCC ACCCTGAGTG TCAGCCCCAG 1860

AATGGCTCAG TGACCTGTTT TGGACCGGAG GCTGACCAGT GTGTGGCCTG TGCCCACTAT 1920

AAGGACCCTC CCTTCTGCGT GGCCCGCTGC CCCAGCGGTG TGAAACCTGA CCTCTCCTAC 1980

ATGCCCATCT GGAAGTTTCC AGATGAGGAG GGCGCATGCC AGCCTTGCCC CATCAACTGC 2040

ACCCACTCCT GTGTGGACCT GGATGACAAG GGCTGCCCCG CCGAGCAGAG AGCCAGCCCT 2100

CTGACGTCCA TCGTCTCTGC GGTGGTTGGC ATTCTGCTGG TCGTGGTCTT GGGGGTGGTC 2160

TTTGGGATCC TCATCAAGCG ACGGCAGCAG AAGATCCGGA AGTACACGAT GCGGAGACTG 2220

CTGCAGGAAA CGGAGCTGGT GGAGCCGCTG ACACCTAGCG GAGCGATGCC CAACCAGGCG 2280

CAGATGCGGA TCCTGAAAGA GACGGAGCTG AGGAAGGTGA AGGTGCTTGG ATCTGGCGCT 2340

TTTGGCACAG TCTACAAGGG CATCTGGATC CCTGATGGGG AGAATGTGAA AATTCCAGTG 2400

GCCATCAAAG TGTTGAGGGA AAACACATCC CCCAAAGCCA ACAAAGAAAT CTTAGACGAA 2460

GCATACGTGA TGGCTGGTGT GGGCTCCCCA TATGTCTCCC GCCTTCTGGG CATCTGCCTG 2520

ACATCCACGG TGCAGCTGGT GACACAGCTT ATGCCCTATG GCTGCCTCTT AGACCATGTC 2580

CGGGAAAACC GCGGACGCCT GGGCTCCCAG GACCTGCTGA ACTGGTGTAT GCAGATTGCC 2640

AAGGGGATGA GCTACCTGGA GGATGTGCGG CTCGTACACA GGGACTTGGC CGCTCGGAAC 2700

GTGCTGGTCA AGAGTCCCAA CCATGTCAAA ATTACAGACT TCGGGCTGGC TCGGCTGCTG 2760

GACATTGACG AGACAGAGTA CCATGCAGAT GGGGGCAAGG TGCCCATCAA GTGGATGGCG 2820

CTGGAGTCCA TTCTCCGCCG GCGGTTCACC CACCAGAGTG ATGTGTGGAG TTATGGTGTG 2880
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54

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ACTGTGTGGG AGCTGATGAC TTTTGGGGCC AAACCTTACG ATGGGATCCC AGCCCGGGAG 2940

ATCCCTGACC TGCTGGAAAA GGGGGAGCGG CTGCCCCAGC CCCCCATCTG CACCATTGAT 3000

GTCTACATGA TCATGGTCAA ATGTTGGATG ATTGACTCTG AATGTCGGCC AAGATTCCGG 3060

GAGTTGGTGT CTGAATTCTC CCGCATGGCC AGGGACCCCC AGCGCTTTGT GGTCATCCAG 3120

AATGAGGACT TGGGCCCAGC CAGTCCCTTG GACAGCACCT TCTACCGCTC ACTGCTGGAG 3180

GACGATGACA TGGGGGACCT GGTGGATGCT GAGGAGTATC TGGTACCCCA GCAGGGCTTC 3240

TTCTGTCCAG ACCCTGCCCC GGGCGCTGGG GGCATGGTCC ACCACAGGCA CCGCAGCTCA 3300

TCTACCAGGA GTGGCGGTGG GGACCTGACA CTAGGGCTGG AGCCCTCTGA AGAGGAGGCC 3360

CCCAGGTCTC CACTGGCACC CTCCGAAGGG GCTGGCTCCG ATGTATTTGA TGGTGACCTG 3420

GGAATGGGGG CAGCCAAGGG GCTGCAAAGC CTCCCCACAC ATGACCCCAG CCCTCTACAG 3480

CGGTACAGTG AGGACCCCAC AGTACCCCTG CCCTCTGAGA CTGATGGCTA CGTTGCCCCC 3540

CTGACCTGCA GCCCCCAGCC TGAATATGTG AACCAGCCAG ATGTTCGGCC CCAGCCCCCT 3600

TCGCCCCGAG AGGGCCCTCT GCCTGCTGCC CGACCTGCTG GTGCCACTCT GGAAAGGGCC 3660

AAGACTCTCT CCCCAGGGAA GAATGGGGTC GTCAAAGACG TTTTTGCCTT TGGGGGTGCC 3720

GTGGAGAACC CCGAGTACTT GACACCCCAG GGAGGAGCTG CCCCTCAGCC CCACCCTCCT 3780

CCTGCCTTCA GCCCAGCCTT CGACAACCTC TATTACTGGG ACCAGGACCC ACCAGAGCGG 3840

GGGGCTCCAC CCAGCACCTT CAAAGGGACA CCTACGGCAG AGAACCCAGA GTACCTGGGT 3900

CTGGACGTGC CAGTGTGAAC CAGAAGGCCA AGTCCGCAGA AGCCCTGATG TGTCCTCAGG 3960

GAGCAGGGAA GGCCTGACTT CTGCTGGCAT CAAGAGGTGG GAGGGCCCTC CGACCACTTC 4020

CAGGGGAACC TGCCATGCCA GGAACCTGTC CTAAGGAACC TTCCTTCCTG CTTGAGTTCC 4080

CAGATGGCTG GAAGGGGTCC AGCCTCGTTG GAAGAGGAAC AGCACTGGGG AGTCTTTGTG 4140

GATTCTGAGG CCCTGCCCAA TGAGACTCTA GGGTCCAGTG GATGCCACAG CCCAGCTTGG 4200

CCCTTTCCTT CCAGATCCTG GGTACTGAAA GCCTTAGGGA AGCTGGCCTG AGAGGGGAAG 4260

CGGCCCTAAG GGAGTGTCTA AGAACAAAAG CGACCCATTC AGAGACTGTC CCTGAAACCT 4320

AGTACTGCCC CCCATGAGGA AGGAACAGCA ATGGTGTCAG TATCCAGGCT TTGTACAGAG 4380

TGCTTTTCTG TTTAGTTTTT ACTTTTTTTG TTTTGTTTTT TTAAAGACGA AATAAAGACC 4440

CAGGGGAGAA TGGGTGTTGT ATGGGGAGGC AAGTGTGGGG GGTCCTTCTC CACACCCACT 4500

TTGTCCATTT GCAAATATAT TTTGGAAAAC                                  4530
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(2) INFORMATION FOR SEQ ID NO:10:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 757 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single

55

        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCCGGGGGTC CTGGAAGCCA CAAGGTAAAC ACAACACATC CCCCTCCTTG ACTATGCAAT   60
TTTACTAGAG GATGTGGTGG GAAAACCATT ATTTGATATT AAAACAAATA GGCTTGGGAT   120

GGAGTAGGAT GCAAGCTCCC CAGGAAAGTT TAAGATAAAA CCTGAGACTT AAAAGGGTGT   180

TAAGAGTGGC AGCCTAGGGA ATTTATCCCG GACTCCGGGG GAGGGGGCAG AGTCACCAGC   240

CTCTGCATTT AGGGATTCTC CGAGGAAAAG TGTGAGAACG GCTGCAGGCA ACCCAGGCGT   300

CCCGGCGCTA GGAGGGACGA CCCAGGCCTG CGCGAAGAGA GGGAGAAAGT GAAGCTGGGA   360

GTTGCCGACT CCCAGACTTC GTTGGAATGC AGTTGGAGGG GGCGAGCTGG GAGCGCGCTT   420

GCTCCCAATC ACAGGAGAAG GAGGAGGTGG AGGAGGAGGG CTGCTTGAGG AAGTATAAGA   480

ATGAAGTTGT GAAGCTGAGA TTCCCCTCCA TTGGGACCGG AGAAACCAGG GGAGCCCCCC   540

GGGCAGCCGC GCGCCCCTTC CCACGGGGCC CTTTACTGCG CCGCGCGCCC GGCCCCCACC   600

CCTCGCAGCA CCCCGCGCCC CGCGCCCTCC CAGCCGGGTC CAGCCGGAGC CATGGGGCCG   660

GAGCCGCAGT GAGCACCATG GAGCTGGCGG CCTTGTGCCG CTGGGGGCTC CTCCTCGCCC   720

TCTTGCCCCC CGGAGCCGCG AGCACCCAAG GTGGGTC   757

(2) INFORMATION FOR SEQ ID NO:11:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 539 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCCGGGGGTC CTGGAAGCCA CAAGGTAAAC ACAACACATC CCCCTCCTTG ACTATCAATT   60

TTACTAGAGG ATGTGGTGGG AAAACCATTA TTTGATATTA AAACAAATAG GCTTGGGATG   120

GAGTAGGATG CAAGCTCCCA GGAAAGTTTA AGATAAAACC TGAGACTTAA AAGGGTGTTA   180

AGAGTGGCAG CCTAGGGAAT TTATCCCGGA CTCCGGGGGA GGGGGCAGAG TCACCAGCCT   240

CTGCATTTAG GGATTCTCCG AGGAAAAGTG TGAGAACGGC TGCAGGCAAC CCAGCTTCCC   300

GGCGCTAGGA GGGACGCACC CAGGCCTGCG CGAAGAGAGG GAGAAAGTGA AGCTGGGAGT   360

TGCCACTCCC AGACTTGTTG GAATGCAGTT GGAGGGGGCG AGCTGGGAGC GCGCTTGCTC   420

CCAATCACAG GAGAAGGAGG AGGTGGAGGA GGAGGGCTGC TTGAGGAAGT ATAAGAATGA   480

AGTTGTGAAG CTGAGATTCC CCTCCATTGG GACCGGAGAA ACCAGGGAGC CCCCCGGG   539

(2) INFORMATION FOR SEQ ID NO:12:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 1717 base pairs

56

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        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAATTCGGCA CGAGTACAGA AGGTAAAGGC TGTCTCTATG GAGCCACTGG CCATCCTGGT   60

GCTGCTGTGC TTTCCGATCT GCTCAGCATA TCCTCTGCAT GGGGCAGTGA GACAAGACCA  120

CTCAACCATG GATCTTGCTC AGCAATACCT AGAAAAATAC TACAACTTTA GAAAAAATGA  180

GAAACAATTT TTCAAAAGAA AGGACAGTAG TCCTGTTGTC AAAAAAATTG AAGAAATGCA  240

GAAGTTCCTT GGGCTGGAGA TGACAGGGAA GCTGGACTCG AACACTGTGG AGATGATGCA  300

CAAGCCCCGG TGTGGTGTTC CCGACGTTGG TGGCTTCAGT ACCTTTCCAG GTTCACCCAA  360

ATGGAGGAAA AACCACATCT CCTACAGGAT TGTGAATTAT ACACTGGATT TACCAAGAGA  420

GAGTGTGGAT TCTGCCATTG AGAGAGCTTT GAAGGTCTGG GAGGAGGTGA CCCCACTCAC  480

ATTCTCCAGG ATCTCTGAAG GAGAGGCTGA CATAATGATC TCCTTTGCAG TTGGAGAACA  540

TGGAGACTTT TACCCTTTTG ATGGAGTGGG ACAGAGCTTG GCTCATGCCT ACCCACCTGG  600

CCCTGGATTT TATGGAGATG CTCACTTCGA TGATGATGAG AAATGGTCAC TGGGACCCTC  660

AGGGACCAAT TTATTCCTGG TTGCTGCGCA TGAACTTGGT CACTCCCTGG GTCTCTTTCA  720

CTCAAACAAC AAAGAATCTC TGATGTACCC AGTCTACAGG TTCTCCACGA GCCAAGCCAA  780

CATTCGCCTT TCTCAGGATG ATATAGAGGG CATTCAATCC CTGTATGGAG CCCGCCCCTC  840

CTCTGATGCC ACAGTGGTTC CTGTGCCCTC TGTCTCTCCA AAACCTGAGA CCCCAGTCAA  900

ATGTGATCCT GCTTTGTCCT TTGATGCAGT CACCATGCTG AGAGGGGAAT TCCTATTCTT  960

TAAAGACAGG CACTTCTGGC GTAGAACCCA GTGGAATCCC GAGCCTGAAT TCCATTTGAT 1020

TTCAGCATTT TGGCCCTCTC TTCCTTCAGG CTTAGATGCT GCCTATGAGG CAAATAACAA 1080

GGACAGAGTT CTGATTTTTA AAGGAAGTCA GTTCTGGGCA GTCCGAGGAA ATGAAGTCCA 1140

AGCAGGTTAC CCAAAGAGGA TCCACACTCT TGGCTTTCCT CCCACCGTGA AGAAGATTGA 1200

TGCAGCTGTT TTTGAAAAGG AGAAGAAGAA GACGTATTTC TTTGTAGGTG ACAAATACTG 1260

GAGATTTGAT GAGACAAGAC AGCTTATGGA TAAAGGCTTC CCGAGACTGA TAACAGATGA 1320

CTTCCCAGGA ATTGAGCCAC AAGTTGATGC TGTGTTACAT GCATTTGGGT TTTTTTATTT 1380

CTTCTGTGGA TCATCACAGT TCGAGTTTGA CCCCAATGCC AGGACGGTGA CACACACACT 1440

GAAGAGCAAC AGCTGGCTGT TGTGCTGATT ATCATGATGA CAAGACATAT ACAACACTGT 1500

AAAATAGTAT TTCTCGCCTA ATTTATTATG TGTCATAATG ATGAATTGTT CCTGCATGTG 1560
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57

CTGTGGCTCG AGATGAGCCC AGCAGATAGA TGTCTTTCTT AATGAACCAC AGAGCATCAC 1620

CTGAGCACAG AAGTGAAAGC TTCTCGGTAC ACTAGGTGAG AGGATGCATC CCCATGGGTA 1680

CTTTATTGTT TAATAAAGAA CTTTATTTTT GAACCAT   1717

(2) INFORMATION FOR SEQ ID NO:13:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 650 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GATATCAAGA GGGTGATGCA AACGTCCCAG GAGTGTTCAA GATAAAACCG GAGACTGCAA   60

AGACGGGTAA AGGGATGCTG TGCTTTTAGG AAGTGGATGA GAACTGCAAG CAAGCAAGCA   120

AGCAAGCAAG CAAGCAAGCA AGCAAGCAAG CAAGCAAGCT AGGCGTCGGG GCACAGGGCA   180

GGCGCACCCA GGCCTGCGCC GGGAGGGAGA AAGTGAAAGC TGGGAGCAGC CACTCCCAGT   240

CTTGCTGGAA TGCAGTTGGA GGGGTGGGGG GGCGAGCCGA GAGCGCGCGG CTGCCAATCA   300

CGGGCGGAGG AGGAGGTGGA GGAGGAGGGC TGCTCGAGGA AGTGCGGCGT GAAGTTGTGG   360

AGCTGAGATT GCCCGCCGCT GGGGACCCGG AGCCCAGGAG CGCCCCTTCC CAGGCGGCCC   420

CTTCCGGCGC CGGCCTGTGC CTGCCCTCGC CGCGCCCCCC GCGCCCGCAG CCTGGTCCAG   480

CCTGAGCCAT GGGGCCGGAG CCGCAATGAT CATCATGGAG CTGGCGGCCT GGTGCCGCTG   540

GGGGTTCCTC CTCGCCCTCC TGCCCCCCGG AATCGCGGGC ACCCAAGGTG GGTCTTGGCT   600

TGGGAAGGGC TCTGGCCGCT GTGCTGCCCA CGGGCCGGAG CGCGGAGCTC   650

(2) INFORMATION FOR SEQ ID NO:14:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 3955 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCGGGCCGGA GCCGCAATGA TCATCATGGA GCTGGCGGCC TGGTGCCGCT GGGGGGTTCCT   60

CCTCGCCCTC CTGCCCCCCG GAATCGCGGG CACCCAAGTG TGTACCGGCA CAGACATGAA   120

GTTGCGGCTC CCTGCCAGTC CTGAGACCCA CCTGGACATG CTCCGCCACC TGTACCAGGG   180

CTGTCAGGTA GTGCAGGGCA ACTTGGAGCT TACCTACGTG CCTGCCAATG CCAGCCTCTC   240

ATTCCTGCAG GACATCCAGG AAGTTCAGGG TTACATGCTC ATCGCTCACA ACCAGGTGAA   300

GCGCGTCCCA CTGCAAAGGC TGCGCATCGT GAGAGGGACC CAGCTCTTTG AGGACAAGTA   360

TGCCCTGGCT GTGCTAGACA ACCGAGATCC TCAGGACAAT GTCGCCGCCT CCACCCCAGG   420

58

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CAGAACCCCA GAGGGGCTGC GGGAGCTGCA GCTTCGAAGT CTCACAGAGA TCCTGAAGGG  480

AGGAGTTTTG ATCCGTGGGA ACCCTCAGCT CTGCTACCAG GACATGGTTT TGTGGAAGGA  540

CGTCTTCCGC AAGAATAACC AACTGGCTCC TGTCGATATA GACACCAATC GTTCCCGGGC  600

CTGTCCACCT TGTGCCCCCG CCTGCAAAGA CAATCACTGT TGGGGTGAGA GTCCGGAAGA  660

CTGTCAGATC TTGACTGGCA CCATCTGTAC CAGTGGTTGT GCCCGGTGCA AGGGCCGGCT  720

GCCCACTGAC TGCTGCCATG AGCAGTGTGC CGCAGGCTGC ACGGGCCCCA AGCATTCTGA  780

CTGCCTGGCC TGCCTCCACT TCAATCATAG TGGTATCTGT GAGCTGCACT GCCCAGCCCT  840

CGTCACCTAC AACACAGACA CCTTTGAGTC CATGCACAAC CCTGAGGGTC GCTACACCTT  900

TGGTGCCAGC TGCGTGACCA CCTGCCCCTA CAACTACCTG TCTACGGAAG TGGGATCCTG  960

CACTCTGGTG TGTCCCCCGA ATAACCAAGA GGTCACAGCT GAGGACGGAA CACAGCGTTG 1020

TGAGAAATGC AGCAAGCCCT GTGCTCGAGT GTGCTATGGT CTGGGCATGG AGCACCTTCG 1080

AGGGGCGAGG GCCATCACCA GTGACAATGT CCAGGAGTTT GATGGCTGCA AGAAGATCTT 1140

TGGGAGCCTG GCATTTTTGC CGGAGAGCTT TGATGGGGAC CCCTCCTCCG GCATTGCTCC 1200

GCTGAGGCCT GAGCAGCTCC AAGTGTTCGA AACCCTGGAG GAGATCACAG GTTACCTGTA 1260

CATCTCAGCA TGGCCAGACA GTCTCCGTGA CCTCAGTGTC TTCCAGAACC TTCGAATCAT 1320

TCGGGGACGG ATTCTCCACG ATGGCGCGTA CTCATTGACA CTGCAAGGCC TGGGGATCCA 1380

CTCGCTGGGG CTGCGCTCAC TGCGGGAGCT GGGCAGTGGA TTGGCTCTGA TTCACCGCAA 1440

CGCCCATCTC TGCTTTGTAC ACACTGTACC TTGGGACCAG CTCTTCCGGA ACCCACATCA 1500

GGCCCTGCTC CACAGTGGGA ACCGGCCGGA AGAGGACTTG TGCGTCTCGA GCGGCTTGGT 1560

CTGTAACTCA CTGTGTGCCC ACGGGCACTG CTGGGGGCCA GGGCCCACCC AGTGTGTCAA 1620

CTGCAGTCAT TTCCTTCGGG GCCAGGAGTG TGTGGAGGAG TGCCGAGTAT GGAAGGGGCT 1680

CCCCCGGGAG TATGTGAGTG ACAAGCGCTG TCTGCCGTGT CACCCCGAGT GTCAGCCTCA 1740

AAACAGCTCA GAGACCTGCT TTGGATCGGA GGCTGATCAG TGTGCAGCCT GCGCCCACTA 1800

CAAGGACTCG TCCTCCTGTG TGGCTCGCTG CCCCAGTGGT GTGAAACCGG ACCTCTCCTA 1860

CATGCCCATC TGGAAGTACC CGGATGAGGA GGGCATATGC CAGCCGTGCC CCATCAACTG 1920

CACCCACTCC TGTGTGGATC TGGATGAACG AGGCTGCCCA GCAGAGCAGA GAGCCAGCCC 1980

GGTGACATTC ATCATTGCAA CTGTAGAGGG CGTCCTGCTG TTCCTGATCT TAGTGGTGGT 2040

CGTTGGAATC CTAATCAAAC GAAGGAGACA GAAGATCCGG AAGTATACGA TGCGTAGGCT 2100

GCTGCAGGAA ACTGAGTTAG TGGAGCCGCT GACGCCCAGC GGAGCAATGC CCAACCAGGC 2160

TCAGATGCGG ATCCTAAAAG AGACGGAGCT AAGGAAGGTG AAGGTGCTTG GATCAGGAGC 2220

TTTTGGCACT GTCTACAAGG GCATCTGGAT CCCAGATGGG GAGAATGTGA AAATCCCCGT 2280

GGCTATCAAG GTGTTGAGAG AAAACACATC TCCTAAAGCC AACAAAGAAA TTCTAGATGA 2340
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AGCGTATGTG ATGGCTGGTG TGGGTTCTCC GTATGTGTCC CGCCTCCTGG GCATCTGCCT 2400

GACATCCACA GTACAGCTGG TGACACAGCT TATGCCCTAC GGCTGCCTTC TGGACCATGT 2460

CCGAGAACAC CGAGGTCGCC TAGGCTCCCA GGACCTGCTC AACTGGTGTG TTCAGATTGC 2520

CAAGGGGATG AGCTACCTGG AGGACGTGCG GCTTGTACAC AGGGACCTGG CTGCCCGGAA 2580

TGTGCTAGTC AAGAGTCCCA ACCACGTCAA GATTACAGAT TTCGGGCTGG CTCGGCTGCT 2640

GGACATTGAT GAGACAGAGT ACCATGCAGA TGGGGGCAAG GTGCCCATCA AATGGATGGC 2700

ATTGGAATCT ATTCTCAGAC GCCGGTTCAC CCATCAGAGT GATGTGTGGA GCTATGGAGT 2760

GACTGTGTGG GAGCTGATGA CTTTTGGGGC CAAACCTTAC GATGGAATCC CAGCCCGGGA 2820

GATCCCTGAT TTGCTGGAGA AGGGAGAACG CCTACCTCAG CCTCCAATCT GCACCATTGA 2880

TGTCTACATG ATTATGGTCA AATGTTGGAT GATTGACTCT GAATGTCGCC CGAGATTCCG 2940

GGAGTTGGTG TCAGAATTTT CACGTATGGC GAGGGACCCC CAGCGTTTTG TGGTCATCCA 3000

GAACGAGGAC TTGGGCCCAT CCAGCCCCAT GGACAGTACC TTCTACCGTT CACTGCTGGA 3060

AGATGATGAC ATGGGTGACC TGGTAGACGC TGAAGAGTAT CTGGTGCCCC AGCAGGGATT 3120

CTTCTCCCCG GACCCTACCC CAGGCACTGG GAGCACAGCC CATAGAAGGC ACCGCAGCTC 3180

GTCCACCAGG AGTGGAGGTG GTGAGCTGAC ACTGGGCCTG GAGCCCTCGG AAGAAGGGCC 3240

CCCCAGATCT CCACTGGCTC CCTCGGAAGG GGCTGGCTCC GATGTGTTTG ATGGTGACCT 3300

GGCAATGGGG GTAACCAAAG GGCTGCAGAG CCTCTCTCCA CATGACCTCA GCCCTCTACA 3360
GCGGTACAGC GAGGACCCCA CATTACCTCT GCCCCCCGAG ACTGATGGCT ATGTTGCTCC 3420

CCTGGCCTGC AGCCCCCAGC CCGAGTATGT GAACCAATCA GAGGTTCAGC CTCAGCCTCC 3480

TTTAACCCCA GAGGGTCCTC TGCCTCCTGT CCGGCCTGCT GGTGCTACTC TAGAAAGACC 3540

CAAGACTCTC TCTCCTGGGA AGAATGGGGT TGTCAAAGAC GTTTTTGCCT TCGGGGGTGC 3600

TGTGGAGAAC CCTGAATACT TAGTACCGAG AGAAGGCACT GCCTCTCCGC CCCACCCTTC 3660

TCCTGCCTTC AGCCCAGCCT TTGACAACCT CTATTACTGG GACCAGAACT CATCGGAGCA 3720

GGGGCCTCCA CCAAGTAACT TTGAAGGGAC CCCCACTGCA GAGAACCCTG AGTACCTAGG 3780

CCTGGATGTA CCTGTATGAG ACGTGTGCAG ACGTCCTGTG CTTTCAGAGT GGGGAAGGCC 3840

TGACTTGTGG TCTCCATCGC CACAAAGCAG GGAGAGGGTC CTCTGGCCAC ATTACATCCA 3900

GGGCAGACGG CTCTACCAGG AACCTGCCCC GAGGAACCTT TCCTTGCTGC TTGAA      3955
```

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 721 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

60

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GATATCCCAG AGAGTCTTGG AAGTCACCAG TTAGACATAA CACATTCCCT TCCCAGGCTG    60

ATTTTACCTG AGGATGTGGC GACAAACCCA TTATCTGGTA TTAAGAGTGT GATGCAAACG   120

TTCCAAGAGT ATCCAAGATA AAACCCACCC AAGACTGCAA AGAGGGGTAA AGAGATGCCC   180

TGCTTTTAGG AAGTGGGTGA GAACTGCAAG CAAGCAAGCA AGCGAGGCGT CAGGGCACAG   240

CGCGACGCAC CCAGCCTGCG CCGGGAGGGA GAAAGTGAAG CTGGGAGCAG CCACTCCCAG   300

TCTTGCTGGA AGTCAGTTGG AGGGGTGGGG GGGCGAGCCG GGAGCGCGCG GCTCCCAATC   360

ACGGGCGGCG GAGGAGGCGG AGGAGGAGGG CTGCTCGAGG AAGTGCGGCG TGAAGTTGTG   420

GAGCTGAGAT TGCCCGCCGC TGGGGACCCG GAGCCCAGGA GCGCCCCTTC CCAGGCGGCC   480

CCTTCCGGCG CCGCGCCTGT GCCTGCCCTC GCCGCGCCCC GGCCCGCAGC CTGGTCCAGC   540

CTGAGCCATG GGGCCGGAGC CGCAGTGATC ATCATGGAGC TGGCGGCCTG GTGCCGTTGG   600

GGGTTCCTCC TCGCCCTCCT GTCCCCCGGA GCCGCGGGTA CCCAAGGTGG GTCTTGGCTT   660

GGGGAGGGCT CGGGCCGCTA CGCTGCCCAC GGCGGCCGGA GCCGCGGGGC CCCGAGAGCT   720

C                                                                  721
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61

What is claim d is:

1.   A purified protein designated HPBF which binds to the promoter region of the ERBB2 gene and has a molecular weight of about 44,000-47,000 daltons as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions and which comprises the amino acid sequence of SEQ ID NOS: 1 and 2.

2.   A purified antibody which specifically binds the protein of Claim 1.

3.   The antibody of Claim 2, wherein the antibody is conjugated to a therapeutic drug.

4.   The antibody of Claim 2, wherein the antibody is conjugated to a detectable moiety.

5.   The antibody of Claim 2, wherein the antibody is bound to a solid support.

6.   A bioassay for determining the amount of HPBF in a biological sample comprising:

       a)   contacting the biological sample with a nucleic acid to which the HPBF binds under conditions such that an HPBF/nucleic acid complex can be formed; and

       b)   determining the amount of the HPBF/nucleic acid complex, the amount of the complex indicating the amount of HPBF in the sample.

7.   The bioassay of Claim 6, wherein the nucleic acid is the nucleic acid set forth in SEQ ID NO:3.

8.   A bioassay for determining the amount of HPBF in a biological sample comprising:
       a)   contacting the biological sample with an antibody under conditions such

62

that a specific complex of the antibody and HPBF can be formed; and

      b)   determining the amount of the antibody/HPBF complex, the amount of the complex indicating the amount of HPBF in the biological sample.

9.   A method of detecting the presence of a cancer in a subject comprising determining the presence of a detectable amount of HPBF in a biopsy from the subject, the presence of a detectable amount of HPBF relative to the absence of HPBF in a normal control indicating the presence of a cancer.

10.   A method of determining the prognosis of a subject having cancer comprising determining the presence of a detectable amount of HPBF in a biopsy from the subject, the presence of a detectable amount of HPBF relative to the absence of HPBF in a normal control indicating a decreased chance of long-term survival.

11.   A DNA isolate encoding the protein of Claim 1.

12.   A bioassay for screening substances for the ability to inhibit the activity of HPBF comprising:

      a)   administering the substance to a cell construct comprising:

      i)

the promoter region of ERBB2 linked to a reporter gene; and

      ii)

an activated gene encoding HPBF;

      b)   determining the amount of the reporter gene product; and

      c)   selecting those substances which inhibit the expression of the reporter gene product.

13.   A bioassay for screening substances for the ability to inhibit the mitogenic activity of HPBF in NIH3T3 cells, comprising:

      a)   administering the substance to the cells;

      b)   administering HPBF to the cells;

63

    c)   determining the mitogenic activity of HPBF in the substance-treated cells; and

    d)   selecting those substances which inhibit the mitogenic activity of HPBF in the cells.

14.   A bioassay for screening substances for the ability to inhibit the production of HPBF, comprising:

    a)   administering the substance to a cell having an activated gene encoding HPBF;

    b)   determining the amount of HPBF produced; and

    c)   selecting those substances which inhibit the production of HPBF.

15.   A method of inhibiting a biological activity mediated by HPBF comprising preventing the HPBF from binding to the promoter region of the ERBB2 gene sequence.

16.   The method of Claim 15, wherein the binding to the promoter region is prevented by an antisense nucleotide sequence.

17.   The method of Claim 15, wherein the binding to the promoter region is prevented by a nongenomic nucleic acid sequence to which the HPBF binds.
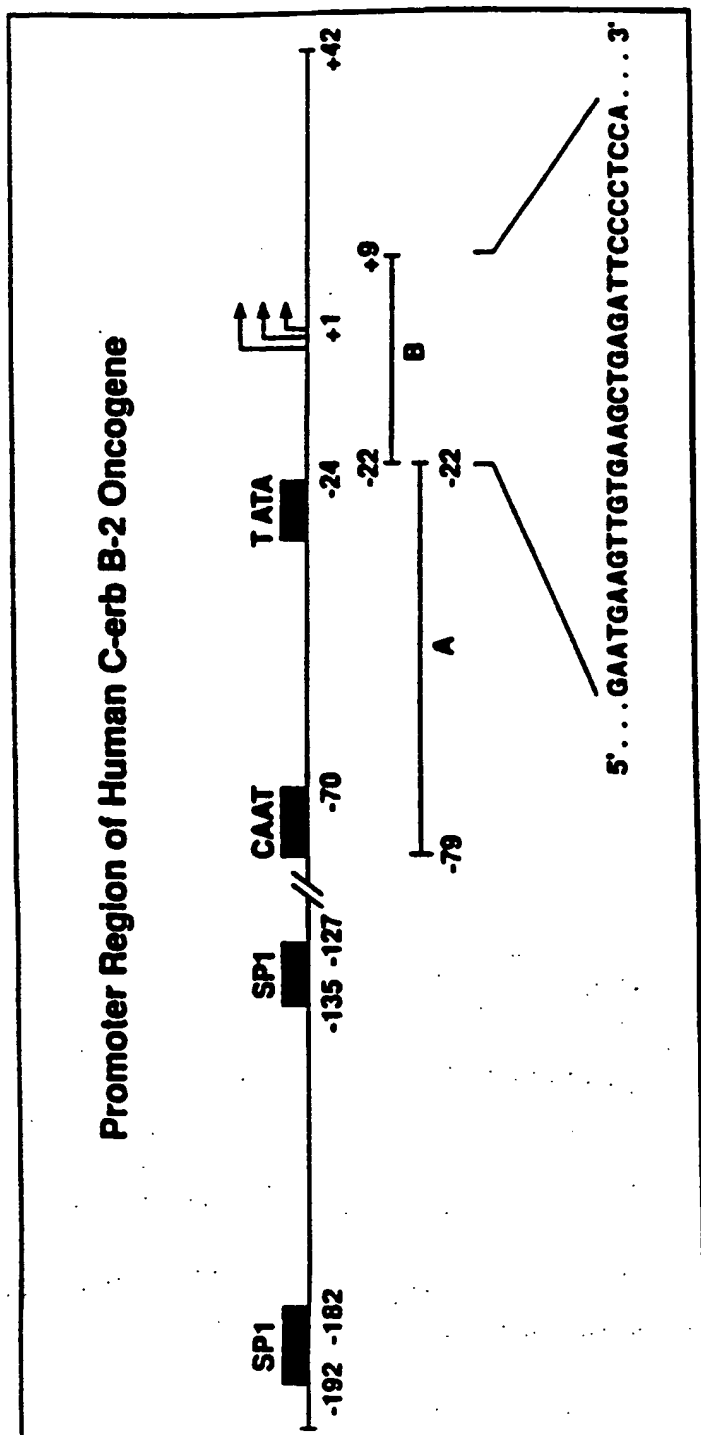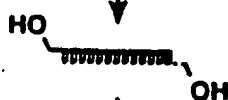
1/2

Fig-1
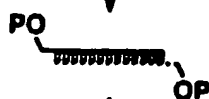
STRATEGY FOR A SEQUENCE - SPECIFIC DNA-SEPHAROSE RESIN

5'...TACGAATGAAGTTGTGAAGCTGAGATTCCCCTCC...3'

3'...CTTACTTCAACACTTCGACTCTAAGGGGAGGCAT...5'

B (-22/+9)   1

Annealing of Complementary Oligonucleotides

HO
OH

2

Phosphorylation of 5'-Protruding Ends With T4 Kinase and ATP

PO
OP

3

Polymerization of the Complementary Oligonucleotides With T4 DNA Ligase and ATP

4

Coupling of the Ligated DNA
to the Activated Sepharose

CNBr-Activated
Sepharose 4B

Affinity Resin

Fig-2

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/12   C07K14/47   C07K16/18   G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO-A-92 20798 (GENETECH, INC.) 26 November 1992 ---	
A	EP-A-0 505 148 (YEDA RESEARCH AND DEVELOPMENT COMPANY LIMITED) 23 September 1992 ---	
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 16, 22 April 1994 BALTIMORE,USA, pages 12285-12289, SARKAR ET AL 'C-ERBB-2 PROMOTER-SPECIFIC DNA-BINDING PROTEIN ISOLATED FROM HUMAN BREAST CANCER TISSUES DISPLAYS MITOGENIC ACTIVITY' see the whole document -----	1-17

☐ Further documents are listed in the continuation of box C.   ☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 July 1995	1 0.08.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Sitch, W

Form PCT/ISA/210 (second sheet) (July 1992)

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:
   Remark: Although claims 15-17(partially in so far as an in vivo method is concered) are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                           [ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9220798	26-11-92	US-A-	5367060	22-11-94
		AU-B-	659863	01-06-95
		AU-A-	2347492	30-12-92
		EP-A-	0586607	16-03-94
		JP-T-	6508031	14-09-94
EP-A-505148	23-09-92	AU-A-	1298392	24-09-92
		NZ-A-	242000	25-03-94